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Diatoms as an indicator of pharmaceutical contamination in a freshwater system

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DIATOMS AS AN INDICATOR OF PHARMACEUTICAL CONTAMINATION IN A FRESHWATER SYSTEM

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Abstract

Historically, microalgae have been used as bio-indicators of aquatic health and this can be seen in several studies across the world. Often at the bottom of the food chain, microalgae constitute the primary producer of energy of many freshwater ecosystems. Diatoms, a group of microalgae, have been shown to exhibit extreme sensitivity to varying environmental parameters thereby making them as an excellent candidate for studying the impacts of pollutants such as Pharmaceuticals and Personal Care Products (PPCPs). The contamination of PPCPs is common in our inland water bodies with a potential to get into our drinking water supply. This study explores 1. The single and mixture effects of PPCPs on two isolated microalgal species belonging to the diatom community. 2. The presence of PPCPs (Ibuprofen, Estrone, and Triclosan) in the nearshore waters of Lake Simcoe and their impacts on algal community. The laboratory studies consisted of assessment of the toxicological effects of the three PPCPs (Ibuprofen, 17-β Estradiol, and Triclosan) on two diatom species, *Asterionella formosa* and *Diatoma tenuis*, by performing growth inhibition tests. The field component involved assessment of several environmental and algal parameters in the surrounding areas of three Waste Water Treatment Plants (WWTPs) that discharge their effluents via a creek to Lake Simcoe. The results indicated that 1. The toxicological values of PPCPs on the two diatom species varied amongst one another. 2. The combined effects of PPCPs were higher than the effects of single toxicity. 3. PPCP contamination is prevalent in the water outside of WWTPs (as far as the point of confluence with Lake Simcoe). 4. The algal parameters varied according to the presence of PPCPs in the surface waters. 5. Estrone exhibited negative effects on the diatom community.

Keywords: algae, diatoms, PPCPs, Lake Simcoe

Lay Summary

The focus of this study is on the suitability of diatoms as a bio-indicator of Pharmaceuticals and Personal Care Product (PPCP) contamination in Lake Simcoe. This study is undertaken to draw the connection between aquatic life and their environmental parameters. Historically, the dynamics of the microalgae community have been a useful tool for assessing aquatic health due to their sensitivity to changes in the environment. Diatoms are unique in structure and morphology, and as such make an excellent candidate for indicating areas of PPCP contamination. This study investigated three research questions. 1. Is diatom composition influenced by the presence of three PPCPs (Ibuprofen, Estrone, and Triclosan) in this study area? 2. Are the diatom species isolated from these area, *Asterionella formosa* and *Diatoma tenuis*, sensitive to the presence of three PPCPs (Ibuprofen, 17-β Estradiol and Triclosan)? 3. Do the PPCPs in this study cause compounding effects on these species when exposed together than alone? The results showed that diatom composition varied with respect to the presence of three PPCPs, *A. formosa* and *D. tenuis* exhibited different toxicological values to the three PPCPs and, compounding effects of these compounds were more severe than the single compound exposure to these diatom species. This information will assist in the development of a database that outlines the effectiveness of using the diatom community as a tool for assessing aquatic health. In addition, the occurrence of mixture toxicity in our inland waters should be carefully monitored to provide appropriate management strategies of Lake Simcoe.

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List of Tables

Table 2.1 - Predicted toxicological values for individual testing. 45

Table 2.2 - Toxicological values causing x percentage of growth inhibition from mixture toxicity experiment using toxicological values from individual toxicity testing. 50

Table 3.1 – Concentrations in ng/L of PPCPs outside of three WWTPs on Lake Simcoe from data collected during the winter of 2014 and the summer of 2016. 82

Table 3.2 - Dilution factor of Ibuprofen concentrations at each WWTP location. 83

List of Figures

Figure 1.1 – Estra-1,3,5(10)-triene-3,17-diol (17 beta). 11

Figure 1.2 – Benzeneacetic acid, a-methyl-4-(2-methylpropyl). 12

Figure 1.3 – Phenol, 5-chloro-2-(2,4-dichlorophenoxy. 14

Figure 1.4 - A typical colony formation of *Asterionella formosa*. 18

Figure 1.5 - Filamentous colonies of *Diatoma sp.*. 18

Figure 2.1 - Logistic growth curve of *Asterionella formosa.* 25

Figure 2.2 - Logistic growth curve of *Diatoma tenuis.* 25

Figure 2.3 - Specific growth relationship between algal biomass, cell counts, and optical density for *Asterionella formosa*. 26

Figure 2.4 - Specific growth relationship between algal biomass, cell counts, and optical density for *Diatom tenuis*. 27

Figure 2.5 – An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Ibuprofen over the course of the first trial. 28

Figure 2.6 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Ibuprofen over the course of the second trial. 29

Figure 2.7 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Ibuprofen over the course of the third trial. 30

Figure 2.8 – A scatter plot illustration of percentage of growth inhibition with varying Ibuprofen concentrations for *Asterionella formosa*. 31

Figure 2.9 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with 17-β Estradiol over the course of the first trial. 31

Figure 2.10 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with 17-β Estradiol over the course of the second trial. 32
Figure 2.11 - A scatter plot illustration of percentage of growth inhibition with varying 17-β Estradiol concentrations for *Asterionella formosa.* 33
Figure 2.12 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Triclosan over the course of the first trial. 34
Figure 2.13 – An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Triclosan over the course of the second trial. 34
Figure 2.14 - A scatter plot illustration of percentage of growth inhibition with varying Triclosan concentrations for *Asterionella formosa*. 35
Figure 2.15 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Ibuprofen over the course of the first trial. 36
Figure 2.16 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Ibuprofen over the course of the second trial. 37
Figure 2.17 - A scatter plot illustration of percentage of growth inhibition with varying Ibuprofen concentrations for *Diatoma tenuis*. 37
Figure 2.18 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with 17-β Estradiol over the course of the first trial. 38
Figure 2.19 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with 17-β Estradiol over the course of the second trial. 39
Figure 2.20 - A scatter plot illustration of percentage of growth inhibition with varying 17-β Estradiol concentrations for *Diatoma tenuis* 39

Figure 2.21 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Triclosan over the course of the first trial. 40
Figure 2.22 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Triclosan over the course of the second trial..... 41
Figure 2.23 - A scatter plot illustration of percentage of growth inhibition with varying Triclosan concentrations for *Diatoma tenuis.* 41
Figure 2.24 - Semi-log scatter plot of Ibuprofen concentration and percentage inhibition of growth for *Asterionella formosa* using regression analysis. 42
Figure 2.25 - Semi-log scatter plot of 17-b Estradiol concentration and percentage inhibition of growth for *Asterionella formosa* using regression analysis. 43
Figure 2.26 - Semi-log scatter plot of Triclosan concentration and percentage inhibition of growth for *Asterionella formosa* using regression analysis. 43
Figure 2.27 - Semi-log scatter plot of Ibuprofen concentration and percentage inhibition of growth for *Diatoma tenuis* using regression analysis. 44
Figure 2.28 - Semi-log scatter plot of 17-β Estradiol concentration and percentage inhibition of growth for *Diatoma tenuis* using regression analysis. 44
Figure 2.29 - Semi-log scatter plot of Triclosan concentration and percentage inhibition of growth for *Diatoma tenuis* using regression analysis. 45
Figure 2.30 – An illustration of a Blocked ANOVA depicting growth responses of *Asterionella formosa* under exposure to the three PPCPs of study under a range of toxicological exposure... 46
Figure 2.31 - An illustration of a Blocked ANOVA depicting growth responses of *Diatoma tenuis* under exposure to the three PPCPs of study under a range of toxicological exposure. 46

Figure 2.32 – A scatter plot illustration of percentage of growth inhibition with varying mixture toxicity concentrations (ECx) for *Asterionella formosa.* 47

Figure 2.33 – A scatter plot illustration of percentage of growth inhibition with varying mixture toxicity concentrations (ECx) for *Diatoma tenuis*. 48

Figure 2.34 - Semi-log scatter plot of mixture toxicity concentrations (ECx) and percentage inhibition of growth for *Asterionella formosa* using regression analysis. 49

Figure 2.35 - Semi-log scatter plot of mixture toxicity concentrations (ECx) and percentage inhibition of growth for *Diatoma tenuis* using regression analysis. 49

Figure 2.36 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Asterionella formosa* with Ibuprofen..... 51

Figure 2.37 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Asterionella formosa* with 17-β Estradiol. 52

Figure 2.38 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Asterionella formosa* with Triclosan. 53

Figure 2.39 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Diatoma tenuis* with Ibuprofen..... 54

Figure 2.40 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Diatoma tenuis* with 17-β Estradiol..... 55

Figure 2.41 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Diatoma tenuis* with Triclosan. 56

Figure 2.42 - An illustration of a Blocked ANOVA comparing changes in optical density during mixture toxicity testing with *Asterionella formosa.* 57

Figure 2.43 – An illustration of a Blocked ANOVA comparing changes in optical density during mixture toxicity testing with *Diatoma tenuis*..... 58
Figure 3.1 - A map of Lake Simcoe and surrounding municipalities, with each sampling point indicated by the yellow markers. 63
Figure 3.2 – The upstream site at the BWG sampling location. 64
Figure 3.3 – The downstream site at the BWG sampling location. 65
Figure 3.4 – The upstream site at the Orillia sampling location. 66
Figure 3.5 – The downstream site at the Orillia sampling location. 66
Figure 3.6 – The upstream point at the Lagoon City sampling location. 67
Figure 3.7 – The downstream site at the Lagoon City sampling location. 67
Figure 3.8 – The upstream point at the control site sampling location. 68
Figure 3.9 – The downstream point at the control site sampling location. 68
Figure 3.10 - Temperature and pH measurements at each sampling point, where upstream and downstream sites are denoted by ‘1’ and ‘2’. 73
Figure 3.11 - Dissolved oxygen measurements at each sampling site, where upstream and downstream sites are denoted by ‘1’ and ‘2’. 73
Figure 3.12 - Conductivity measurements at each sampling site, where upstream and downstream sites are denoted by ‘1’ and ‘2’. 74
Figure 3.13 - Total suspended solids measurements at each sampling site, where upstream and downstream sites are denoted by ‘1’ and ‘2’. 74
Figure 3.14 - Nutrient concentrations at each sampling site, where upstream and downstream sites are denoted by ‘1’ and ‘2’. A solid horizontal line denotes total phosphorus guideline (Government of Ontario, 2016). 75

Figure 3.15 - PPCP concentrations (ng/L) at the upstream and downstream sites of each sampling location, where upstream and downstream sites are denoted by ‘1’ and ‘2’. 76
Figure 3.16 - Measurements of algal parameters at both points of each sampling location, where upstream and downstream sites are denoted by ‘1’ and ‘2’. 77
Figure 3.17 - Percentage of cells belonging to the diatom community at all sampling sites, where upstream and downstream sites are denoted by ‘1’ and ‘2’. 77
Figure 3.18 - Correlation Matrix plot of environmental and algal variables for the three WWTP sampling locations. 78
Figure 3.19 - Scatter plot illustrating a linear relationship between Estrone concentrations and percentage of cells belonging to the diatom community. 79
Figure 3.20 - RDA ordination triplot of the field data, inclusive of algal species. Sampling sites are denoted by circles; Environmental variables are denoted by red vectors; Algal species are denoted by blue vectors. 80

Contents

Abstract i

Lay Summary ii

Acknowledgements iii

List of Tables iv

List of Figures v

Contents i

1. Introduction 1

1.1 Literature Review 1

1.1.1 PPCP's of emerging concern 1
1.1.2 Drinking Waters 1
1.1.3 Surface Waters 3
1.1.4 Wastewater Effluent 4
1.1.5 PPCPs in Great Lakes 5
1.1.6 PPCPs in Lake Simcoe 9
1.1.7 PPCPs for study 10
1.1.8 Common Indicators of PPCP Contamination 15
1.1.9 Diatoms as a Bioindicator 16
1.1.10 Algal Species for Study 17

1.2 Rationale and Objectives 18

1.2.1 Rationale for the Study 18
1.2.2 Study Objectives 19

2. Individual and Combined Toxicity Testing of Three PPCPs on Diatoms – A Laboratory Study 20

2.1 Introduction 20

2.2 Methodology 20

2.2.1 PPCP Solutions Preparation 21
2.2.2 Culturing Techniques 22
2.2.3 Quantitative Measurements 22
2.2.4 Statistical Analyses 24
2.3 Results 25

2.3.1 Specific Growth Correlations 25
2.3.2 Single Toxicity Testing 27
2.3.3 Mixture Toxicity Testing 45
2.3.4 Optical Density 50
2.4 Discussion 58

2.4.1 Single Toxicity Testing 58
2.4.2 Mixture Toxicity Testing 60
2.4.3 Optical Density 60
2.5 Conclusion 61

3. Investigating the Suitability of Diatoms as an Indicator of PPCP Contamination in Lake Simcoe: A Case Study 62

3.1 Introduction 62

3.2 Methodology 62

3.2.1 Sampling Sites 62
3.2.2 Environmental Parameters 69
3.2.3 Microalgae Analysis 71
3.2.4 Statistical Analyses 71
3.3 Results 72

3.3.1 Environmental Parameters 72
3.3.2 Presence of PPCPs 75
3.3.3 Microalgae Parameters 76
3.3.4 Preliminary Analysis 78
3.3.5 Ordination Analysis 79
3.4 Discussion 81

3.4.1 Toxicological Presence of PPCP Concentrations 81
3.4.2 Implications on Microalgal Composition 84
3.5 Conclusion 85

4. Conclusions 86

References 89

1. Introduction

1.1 Literature Review

1.1.1 PPCP's of emerging concern

Pharmaceuticals and personal care products (PPCPs) entering our freshwater systems have become an emergent issue both on a local and global scale. These contaminants that appear in trace amounts in wastewater effluent and surface waters are finding their way into the drinking water systems (Benotti et al., 2009; Carmona et al., 2014; Daneshvar et al., 2012; Padhye et al., 2014; Rahman et al., 2009; Wray et al., 2014). In addition, the potential impacts of these compounds on the environment, aquatic life, and human life have been sparsely studied and this is a huge concern.

1.1.2 Drinking Waters

Drinking water within Canada is regulated following guidelines set by Health Canada, and the decision to regulate is based on whether, or not the contaminant i) poses health risks of persons, ii) is frequent and abundant in public water systems, and iii) would reduce health risks if properly regulated (US EPA, 2016). In the case of chemical contaminants that are non-carcinogenic but that can cause adverse health effects, a maximum contaminant level goal (MCLG) is recorded and a maximum contaminant level is enforced (US EPA, 2016). In response to the multiple inquiries by various Member States, The World Health Organization (WHO) investigated the potential health impacts of PPCPs in drinking water (World Health Organization, 2012). This report, published in 2012, concluded that adverse human health impacts are not expected to be of concern in drinking waters, as the concentrations seen in published literature and national studies are typically below 50 ng/L (World Health Organization, 2012). Interestingly enough, it is duly noted that to date very few comprehensive, systematic studies in regards to PPCPs in drinking waters on a global scale exist (World Health Organization, 2012).

Since 2012, studies from Europe, Asia, and North America have indicated frequent abundance of various PPCPs in drinking water (Carmona et al., 2014; Daneshvar et al., 2012; Lin et al., 2016; Padhye et al., 2014; Wray et al., 2014; Wu et al., 2014). For instance, a study conducted on the Turia River Basin in Spain found more than a dozen different acidic pharmaceutical compounds including anti-inflammatories, antibiotics, endocrine disruption

compounds (EDCs) and antimicrobial agents, in tap water sources, however, all below 40ng/L (Carmona et al., 2014). Another study, evaluates current and previous values of a wide array of PPCPs in drinking water sources in the Greater Montreal Region. Once again, these contaminants were frequently found, however, at concentrations averaging less than 50ng/L (Daneshvar et al., 2012). An exception of this was Caffeine, which being measured at concentrations greater than 100 ng/L (Daneshvar et al., 2012). It is interesting to note that although the average concentrations of these contaminants typically sit within a low range in drinking water systems, the maximum concentrations measured greatly surpass these values. For example, peak concentrations for two compounds, Naproxen (anti-inflammatory) and Atrazine (herbicide), were found to be 234 and 225 ng/L, respectively (Daneshvar et al., 2012).

Drinking Water Treatment Plants (DWTPs) have recently been studied in regards to their efficacy to remove PPCPs from the source water to the final effluent. Some key processes to reduce PPCPS are ultrafiltration via porous membranes, chlorination, and ozonation (Benotti et al., 2009; Wray et al., 2014). These processes are quite common across North America, particularly in DWTPs that have been either upgraded or built new in the past two decades (Wray et al., 2014). Unfortunately, very few studies exist on this topic. The current understanding is that newer and/or upgraded DWTPs show a reduction in PPCP contaminants (Lin et al., 2016; Padhye et al., 2014). For example, drinking water influent and effluent stations were monitored at an Advanced Drinking Water Treatment Plant (ADWTP) in the Taihu region of China. Various types of PPCPs including antibiotics, beta blockers, antipsychotic drugs, and anti-convulsant drugs were measured in the raw waters (< 40 ng/L), and the treated effluent showed a reduction to less than 2 ng/L for all PPCPs (Lin et al., 2016). Another DWTP was assessed in Southeastern US in terms of PPCP removal from source water to final effluent over the course of one year. Several contaminants were measured in the source water, with Nonylphenol (organic detergent), DEET (pesticide) and Bisphenol-A with highest in concentrations such as 83, 122, and 13 ng/L, respectively (Padhye et al., 2014). This basic DWTP could reduce these concentrations to 12, 20, and 3 ng/L, respectively (Padhye et al., 2014).

Many DWTPs use source water impacted by wastewater, which has led to the recent concerns for PPCPs in drinking water (Benotti et al., 2009). With this information, it would be

safe to suggest that more research pertaining to 1) the frequency and abundance of PPCPs in drinking water and 2) whether, or not the efficiency of current DWTPs is enough to avoid concern for potential risks posed by PPCPs on human health. In order to make sure that our drinking water is safe, it would be wise to adapt to a routine monitoring program on PPCPs in urban drinking water sources.

1.1.3 Surface Waters

PPCP contamination is also appearing in our surface waters from a variety of anthropogenic and domestic sources (Boxall et al., 2012; Dougherty et al., 2010; Hua et al., 2005; Metcalfe et al., 2003; Paíga et al., 2013; Reiss et al, 2002; Wu et al., 2009). Interestingly, some contaminants are known to have an exclusive origin either domestic or agricultural sources and thus, they are being used as chemical tracers for anthropogenic and domestic contamination (Harwood, 2014; Oppenheimer et al., 2012; Young et al., 2008). Anthropogenic sources of PPCP include wastewater effluent as well as leaking septic systems (Oppenheimer et al., 2012; Ternes et al., 2004). PPCPs with long residence time are not efficiently removed in WWTPs; some examples are Sucralose (artificial sweetener) and Acesulfame-K. Other compounds, such as Caffeine and Triclosan (antimicrobial) have also been used effectively as tracer compounds of effluent discharge (Harwood, 2014; Kurissery et al., 2012; Oppenheimer et al., 2012; Young et al., 2008). Few studies exist on the distribution of PPCPs near wastewater effluent discharge locations (Metcalfe et al., 2003). The presence of PPCPs in the aquatic environment depends on flow rate of receiving water body, sedimentation rate, uptake by biota, volatilization, biological degradation, and photodegradation (Blair et al., 2013). Recent literature showed the presence of wastewater effluents as far as 3 km from the source of discharge (Blair et al., 2013; Hua et al., 2005). In addition, many of these contaminants are known to settle into the sediments leading to bioaccumulation by the benthos (Coogan et al., 2007).

One of the major issues of using PPCPs as an anthropogenic marker capable of differentiating domestic and agricultural waste is that PPCPs are now being found adjacent to agricultural fields in similar concentrations of downstream locations of wastewater effluent discharge plants (Topp et al., 2008; Xia et al., 2005). This is largely due to applications of sewage effluent for irrigation, sewage sludge as fertilizer, and vet pharmaceuticals and manure

from livestock (Boxall et al., 2012). Studies pertaining to PPCP contamination in biosolids and sewage effluent for fertilization and irrigation are lacking significantly.

1.1.4 Wastewater Effluent

It is a widely accepted statement that PPCPs are primarily entering our aquatic ecosystems via discharge from wastewater treatment plants (WWTPs) (Li et al., 2010). Although the WWTPs have not been optimized for the said contaminant reduction, incidentally, it does occur. 30-90% of the dose of a drug administered to humans or animals is excreted in urine and feces as a biologically active compound (Metcalfe et al., 2003). These contaminants passing through a typical WWTP in North America may be removed by as much as 98% depending on the contaminant at hand, treatment processes, seasonality, hydrologic parameters, etc. (Daneshvar et al., 2012; Lishman et al., 2006). Unfortunately, the removal efficiency rate of 98% is not the case for most PPCPs. Currently there is a federal regulation in the USA to conduct environmental risk assessments of new pharmaceuticals if their predicted concentration in the effluents is estimated to be greater than 1 μg/L (Federal Drug Administration, 1998). In addition, the Great Lakes Water Quality Agreement of 2012 between Canada and the US, has vouched to regulate any emerging contaminants of concern that may potentially pose threats to public health (Government of Canada, 2012).

The fate and residual concentrations of an individual contaminant is reliant on its volatility, sedimentation, biological degradation, photodegradation, and uptake by biota (e.g. log K_{ow} value) (Blair et al., 2013). Various treatment processes such as physical methods, biodegradation, and chemical advanced oxidation result in the degradation of contaminants (Liu et al., 2009). Other tertiary treatments that have proven to further increase the contaminant removal include sand point filters, activated carbon filters, and lagoon systems providing an area for sedimentation and acting as an additional filtration system. These processes have been shown to be very effective in removing residual concentrations of contaminants in conjunction with the conventional treatment processes (Hollender et al., 2009; Lishman et al., 2006; Reungoat et al., 2010). Seasonality in a temperate area influences the hydrologic parameters, particularly the water temperature. Recently, it has been shown that the temperature and pH play a significant role on the degradation of PPCPs. According to a study in the Greater Montreal Region, lower removal rates of pharmaceuticals were observed in colder seasons and thus correlated with lower

water temperatures (Daneshvar et al., 2012). This information pertaining to degradation of these contaminants, and efficacy of wastewater treatment facilities give a better understanding on how to design an efficient urban wastewater treatment facility.

The available information so far showed a true gap in the research pertaining to the combined sewage overflow (CSO) events. Typically, new and/or recently renovated WWTPs are set-up to avoid CSO events. A recent study suggests that CSO events in conjunction with discharge of WWTPs constitute the main source of environmental release of PPCPs in our aquatic ecosystems (Daneshvar et al., 2012). A recent study from North America suggests that 40-90% of the annual load of hormones and wastewater micropollutants, including PPCPs, that normally would have had relatively high treatment removal efficiency, is contributed by CSO events (Phillips et al., 2012).

One of the major factors that determine the impact of treated effluent on aquatic environment is the method of effluent dispersal (U.S. Geological Survey, 1995). WWTPs typically discharge either via a creek that feeds into a larger water body, or via underground pipes taking the effluents away from the shore to release at the deeper areas of the waterbody. Issues may arise with both these methods if treated sewage is contaminated. For instance, creek discharge may pose great threats to near-shore organisms as the contaminants are most concentrated in a small, shallow, body of water (Coogan et al., 2007; Ferguson, 2012; Reiss et al., 2002). In addition, although the case for discharge via pipe is made on account of substantial dilution and potentially higher mixing, they still can cause significant issues (U.S. Geological Survey, 1995). This includes, the potential for accumulation of contaminants below the thermocline in seasons where low mixing occurs. Although many PPCPs are thought to be short-lived, it must be recognized that their impacts may be just as harmful as that of a long-lasting contaminants (i.e. heavy metals) as the constant influx will ensure their persistence in the environment (Daughton et al., 1999).

1.1.5 PPCPs in Great Lakes

Contaminants of emerging concern (CECs), namely PPCPs, are present in alarming concentrations outside of wastewater treatment facilities in the Great Lakes Basin (Blair et al., 2013; Hull et al., 2015; Li et al., 2010; Metcalfe et al., 2003). This is particularly alarming, as the Great Lakes represent approximately 84% of North America's available freshwater resource

(Blair et al., 2013). In addition to housing a vast array of aquatic organisms, these lakes provide substantial economic benefits, a source for drinking water, and an invaluable resource for industry and agriculture. Therefore, we find many reasons to examine the effects of anthropogenic inputs on this freshwater system. Research in regards to PPCP contamination on the Great Lakes has become quite popular in the last decade.

A series of near-shore water samples were collected and analyzed using a kinetic sampler, known as POCIS (Polar Organic Chemical Integrative Sampler) in order to determine the concentrations of 30 PPCPs, including Endocrine Disrupting Compounds (EDCs), in Lake Ontario, while simultaneously examining the impacts of water temperature and flow on the device, itself (Li et al., 2010). POCIS device is an excellent alternative to other common water samplers as it allows for the determination of time-weighted average concentrations of these contaminants rather than concentrations at an instantaneous moment in time. However, the results of this study do in fact suggest that the concentrations measured from POCIS subsurface samples were consistent with that of the other surface samplers (Li et al., 2010). The samples were collected during the summer of two different years, when a minimum of nine contaminants were detected at each sampling site, with concentrations ranging from 0 - 35 ng/L. Among the highest of the contaminants included were Caffeine, Ibuprofen, and Atenolol (beta blocker).

In 2011, a study conducted in the Hamilton Harbor of Lake Ontario revealed alarming concentrations of four PPCPs: Ibuprofen (anti-inflammatory), Triclosan (antimicrobial), Gemfibrozil (fibrates), and Naproxen (anti-inflammatory) (Csiszar et al., 2011). The objective of this research was to develop a new formulation of the multi-species aquivalence approach to treat ionizing pharmaceuticals as interconverting neutral and ionic forms. Essentially, this model proves to be useful in approximating half-lives and fate of the four aforementioned pharmaceuticals. In doing so, field samples from six stations within the Hamilton Harbor were analyzed for the presence of these PPCPs. These surface and subsurface water samples were collected over the course of one year and revealed that the largest contributor of contamination loading of these pharmaceuticals was likely due to the adjacent WWTP where shallow and narrow waters channels persist, and that the highest concentrations of these compounds were seen in spring more than any other time of the year (Csiszar et al., 2011). Maximum

concentrations of Ibuprofen, Triclosan, Gemfibrozil, and Naproxen were about 1600, 600, 200, and 1200 ng/L, respectively (Csiszar et al., 2011).

Contrary to the most current research of PPCP contamination, a study was conducted within an agricultural landscape that did not receive discharge from WWTPs to assess the impacts from land application of biosolids as soil amendment from a local WWTP, as well as, septic systems on PPCP loading. From this study, it was found that the PPCP contamination in these isolated waters was comparable to that of urban rivers receiving wastewater effluent discharge (Wu et al., 2009). Among the most frequent PPCPs found within this basin were Caffeine, Carbamazepine (antiepileptic), and Paraxanthine (psychoactive stimulant). From this report, it is suggested that PPCPs are being transported into surface waters, likely due to the land application of biosolids, however, more research is required in order to support that septic tanks could be contributing to this pollution significantly (Wu et al., 2009).

A more broad-scale approach was taken to evaluate the distribution and occurrence of multiple PPCPs in the lower Great Lakes region. More specifically, the sampling area encompassed four WWTPs and 100 m increments to a total of 400 m downstream sites of the respective effluent within Lake Ontario, Lake Erie, and the connecting rivers between these lakes. Multiple PPCPs were detected at all four of the WWTP sampling sites in concentrations, usually below 1 µg/L (Metcalfe et al., 2003). The sampling sites located outside of the Little River WWTP were contaminated fairly consistently downstream as far as 400 m (Metcalfe et al., 2003). Whereas, concentrations of PPCPs outside of the West Windsor WWTP became fairly dilute (majority of contaminants below detection limit) within 400 m downstream of the effluent discharge (Metcalfe et al., 2003). It is suspected that this is due to the dilution effect as the effluent of the Little River constitutes the majority of the river that the effluent is being discharged into, as opposed to West Windsor effluent which is discharged into the Detroit River, which in itself is a highly complex hydrologic system. The study showed an overall trend of high PPCP contamination at the closest proximity of the point of discharge of WWTP effluent (Metcalfe et al., 2003).

A risk-based screening approach was conducted in regards to PPCP contamination in the US and Canadian Great Lakes waters. Hazardous Risk Quotients (RQs) were developed for a multitude of PPCPs and CECs, whereby a contaminant could be identified as posing ecological

risk within a certain area. Where the value of RQ is greater than one, it is suspected that the contaminant is high risk for posing risk to the environment (Blair et al., 2013). As previously mentioned, regulations and guidelines in this part of the world, pertaining to this regard are largely lacking. As such, much of the research conducted in efforts of exploring concentrations of varying contaminants serves with little purpose. A comparison of current concentrations of these contaminants to guideline, restrictions, regulations, or benchmarks from across the world, in addition to using previous toxicological data could prove useful in estimating the actual risk of these PPCPs and CECs. This comprehensive study estimated RQs in order of determining the risk of each contaminant in these aquatic systems. Of the 89 chemicals assessed, 37 of them are thought to pose potential risks of being hazardous to the environment at their current concentrations (Hull et al., 2015).

Another study calculated RQ values of more than 30 PPCPs in Lake Michigan at varying proximities outside of several wastewater effluent discharge sites (Blair et al., 2013). Samples collected were taken from both water and sediment, and it was found that 14 PPCPs posed medium to high risk in Lake Michigan and as far as 3.2 km from effluent discharge sites (Blair et al., 2013). Among the most frequently abundant contaminants found in the water and sediment samples were Metformin (antidiabetic), Caffeine, Triclocarbon and Triclosan (Blair et al., 2013).

In summary, the information of PPCP contamination in Great Lakes is quite alarming and should not be taken lightly. As our ability to detect varying compounds at lower concentrations than historically ever possible to detect thus far continues to develop, our recognition of these contaminants in our waters will only grow. These preliminary studies calculating RQ values is a great step in the right direction. Toxicological data varies not only from contaminant to contaminant, but within contaminants, dependent upon the source of literature. Large databases, such as the Ecological Structure Activity Relationships (ECOSAR), seem to be an acceptable resource for identifying toxicological data for various PPCPs, however, this has not become a globally acceptable resource. In addition, it is more common than not, to assess the toxicity of merely one contaminant at a time, when in fact multiple contaminants are being found in the same location at varying concentrations. A recent study suggests that mixture toxicity testing resulting in greater sensitivity of organisms to the toxicity of contaminants, and as such, more consideration should be given to this regard (Geiger, 2014).

1.1.6 PPCPs in Lake Simcoe

Lake Simcoe is a fairly large lake (722 km^2) connected to Lake Ontario and Lake Huron via the Trent Severn waterway. The lake provides a valuable freshwater resource for seven separate municipalities that include over 400,000 residents. It provides suitable habitat for more than 50 species of fish, which plays a major contributing role to the local economy. Lake Simcoe generates more than 200 million dollars for local economy on an annual basis, 80% being attributed to ice fishing alone. Due to the grave significance of this waterbody (eg. industry, recreation, agriculture, etc.), a lot of efforts are required to properly manage Lake Simcoe.

In the past, eutrophication has been a major issue with Lake Simcoe and has led to the recruitment failure of many cold-water fishes (Winter et al., 2011). In addition, over the past few decades we have seen many changes of algal composition due to the introduction of invasive species (Winter et al., 2011). Most recently, the occurrence of PPCP contamination in this freshwater system has become a topic of concern.

A recent study showed evidence of organic micropollutants – including PPCPs – being present in both the influent and effluent of a wastewater treatment plant discharging its effluent into Lake Simcoe (Wray et al., 2014). The major focus of this study was to analyze the impacts of surface shear stress on retention of organic micropollutants, it also suggested the presence of PPCPs such as Acetaminophen, Diclofenac and Naproxen (anti-inflammatories) (Wray et al., 2014). The retention of these contaminants was not 100% and as such, more research would be required to know whether or not they are making their way into Lake Simcoe, and at what concentrations.

Recently, PPCPs have been identified as an emergent issue in the Lake Simcoe Protection Plan and further attention to this topic is thought to be required (Ontario, 2014). A study published in 2014, explored the presence of PPCPs in the influent and effluent of six WWTPs within the Lake Simcoe watershed. Time-weighted average concentration of 13 contaminants was measured using a POCIS, of which 11 of them were found present above their detection limits. Among compounds detected Sucralose and Trimethoprim (antibiotic) were the highest with maximum concentrations of 260 and 99 ng/L, respectively (Metcalfe, 2014). The remaining contaminants were present in fairly low concentrations (< 25 ng/L), which is suspected to be due

to the optimal performances of several advanced wastewater treatment facilities surrounding Lake Simcoe (Metcalfe, 2014).

Outside of these two studies on PPCP contamination into Lake Simcoe, information to this regard is greatly lacking. It would be ideal to have more studies focused on PPCP contamination in wastewater discharge sites, similar to that of the studies on the Great Lakes in order to fully understand the impact of PPCP loading into Lake Simcoe. In addition, Lake Simcoe watershed provides much area for the agricultural industry, and the impacts of biosolids for land amendment on PPCP loading should also be carefully examined.

1.1.7 PPCPs for study

Three PPCPs have been chosen for the present study: 17-β Estradiol, Ibuprofen, and Triclosan. The following is a review of these three compounds, their prevalence in our local systems (Great Lakes Basin and Lake Simcoe), and potential risks that they may pose to our aquatic environments.

1.1.7.1 17-β Estradiol

17-β Estradiol (EST) is a natural phenolic steroid estrogen belonging to the group of Endocrine Disrupting Compounds (EDCs), that is causing some alarm with its prevalence in the freshwater systems (F. Wu et al., 2014). This specific compound is notorious for being a potent EDC that has the potential for disrupting normal sex differentiation and gametogenesis in fish at very low concentrations, especially when compared other natural estrogens (Salomão et al., 2014; F. Wu et al., 2014). The water-octanol partitioning coefficient (log K_{ow}) of EST is 3.9. This indicates a moderate to high ability to combine or dissolve in lipids or fats, rather than in water. Its water solubility is 3.6 mg/L (Z. Liu et al., 2009; US EPA, 2016). This natural estrogen is primarily entering our freshwater systems via livestock, WWTPs, and industrial effluent (Wu et al., 2014). When examining the potential removal of EST or any other EDCs, three processes are looked at: physical removal, biodegradation, and chemical advanced oxidation (Z. Liu et al., 2009).

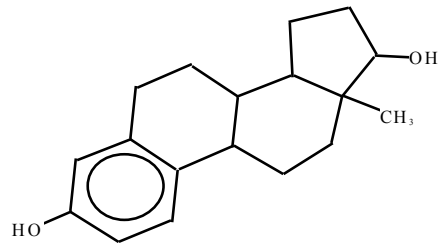

Figure 1.1 – Estra-1,3,5(10)-triene-3,17-diol (17 beta).

Recent studies suggest that most aquatic autotrophs tend to respond quite favorably in the presence of low concentrations of estradiol and have been shown to carry the ability of bioaccumulating estradiol (Julius et al., 2007; Pollock et al., 2015). A study on the diatom, *Melosira varians*, found that concentrations of Estradiol resulted in a larger carrying capacity (~ 200 - 800 μg/L), suggesting that these low concentrations consistently proved to enhance cell growth and physiology (Julius et al., 2007). According to ECOSAR, the EC_{50} value of this compound on green microalgae is 4.278 mg/L (US EPA, 2016). However, other studies examining EC_{50} values of green microalgae reported much lower concentrations. A study on *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus* found $EC_{50\ (96hr)}$ to be 0.87 and 1.07 mg/L, respectively (Salomão et al., 2014). In contrast, a study on the diatom, *Navicula incerta* found an EC_{50} of 10 mg/L, much higher than the acceptable resource from EPA (Liu et al, 2010). Mechanisms of action responsible for cell death in the microalgae community is not yet clearly understood. However, it is seen that these organisms have the ability to uptake and biotransform this compound into less complex forms, such as estriol, which in part causes cell toxicity (Lai et al., 2002)

Larger and more complex organisms have been shown to have high sensitivity to very low concentrations of EST. For instance, studies on the sand goby have shown that concentrations of 97 ng/L significantly impacted male reproductive systems (i.e. maturation), and that a concentration of 669 ng/L greatly increased the mortality in addition to the detrimental impacts on reproduction (i.e. spawning), particularly in males (Wu et al., 2014). In addition, exposure to EST, particularly during early life stages has been suggested to greatly alter sexual differentiation and fecundity in fishes (Wu et al., 2014). Trout are commonly used as indicator

species for various aquatic contaminants due to their increased sensitivity. In regards to EST, rainbow trout have a suggested NOEC of 1 ng/L (Wu et al., 2014).

1.1.7.2 Ibuprofen

Ibuprofen (IBU) is an analgesic belonging to the class of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) (Moro et al., 2014). This PPCP has a log K_{ow} of 3.8, and its water solubility is 21 mg/L (US EPA, 2016). According to ECOSAR, the EC_{50} value of this compound on green microalgae is 41.133 mg/L (US EPA, 2016). Although this value may seem quite high, relative to other PPCPs such as EST, the study of this compound and its fate in our aquatic ecosystems is important because of how prevalent and abundant this PPCP is in Canadian waters (Csiszar et al., 2011; Li et al., 2010). This should come as no surprise, as IBU is among the most frequently used drugs globally, and its use is only increasing with time (Bácsi et al., 2016; Paíga et al., 2013). This compound has been shown to cause morphological and structural alterations to microalgae including cytoplasmic inclusions, which in extreme cases may lead to cellular mortality (Moro et al., 2014). Under ideal conditions, this compound has been shown to be removed with 90-100% efficiency during water treatment processes, however, this is clearly not always the case (Bácsi et al., 2016).

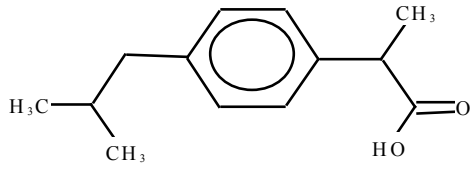

Figure 1.2 – Benzeneacetic acid, a-methyl-4-(2-methylpropyl).

For instance, one study on Lake Ontario revealed that IBU concentrations were reaching as high as 1600 ng/L outside of wastewater effluent discharge (Csiszar et al., 2011). Another study, also on Lake Ontario, collected samples from several sites within the lake at varying locations outside of WWTPs and of 30 PPCPs being measured, IBU was amongst the most frequently and abundantly detected compound (Li et al., 2010). This compound has a great ability to bioaccumulate in an aquatic ecosystem, in addition to being relatively stable against sunlight, and as such its potential effects should not be taken lightly (Moro et al., 2014;

Yamamoto et al., 2009). In addition to WWTP effluent, extremely high concentrations of IBU have been seen in landfill leachate (Paíga et al., 2013).

Alternative resources have also suggested much lower EC_{50} values for this compound with green microalgae, such as 4.01 mg/L and a PNEC of 4.01 µg/L (Pomati et al., 2004). However, the range of toxicological values is highly variable, not only between fish, daphnia, and green microalgae, but also within these organisms. Some studies on microalgae have shown EC_{50} values ranging as high as 340 mg/L, whereas others see this amount of growth inhibition as low as 1 mg/L (Bácsi et al., 2016; Moro et al., 2014). It is also interesting to note, that recent literature has suggested that cyanobacteria may be less susceptible to the toxicity effects of IBU, than that of eukaryotic algae. That is, to say, that potentially IBU loading could result in a reduction of diversity of eukaryotic algae-dominated community assemblages (Bácsi et al., 2016).

Not only are algal communities expected to be impacted by this contaminant, but also organisms from higher trophic levels. IBU pollution is expected to have adverse effects on reproduction of aquatic life (Nesbitt, 2011). This has been well studied in the Florida flagfish, where concentrations above the LOEC of 0.1 µg/L, has been shown to have significant impacts on egg fertilization (Nesbitt, 2011).

1.1.7.3 Triclosan

Triclosan (TRI) is a chlorinated biphenyl ether used as a broad-spectrum antimicrobial and disinfectant agent, commonly found in personal care products such as soaps, detergents, deodorants, toothpastes, etc. (Orvos et al., 2002). The presence of this compound in freshwater systems is of grave concern as it induces toxic effects at fairly low concentrations. TRI is believed to exhibit several mechanisms promoting apoptosis, the most prevalent being the uncoupling of oxidative phosphorylation resulting in cellular mortality (Olanivan et al., 2016, Orvos et al., 2002). This is relatively a stable and non-volatile compound in the environment, with the ability to easily bioaccumulate (Hua et al., 2005; US EPA, 2016; Wang et al., 2013). The log K_{ow} is approximately 4.6, and its water solubility is 10 mg/L (US EPA, 2016). According to ECOSAR, the EC_{50} value for green microalgae for this compound is 1.443 mg/L, however, alternative resources suggest EC_{50} values ranging from 0.7 µg/L to 1.1 mg/L on green microalgae (Franz et al., 2008; Hunt, 2006; Orvos et al., 2002; US EPA, 2016).

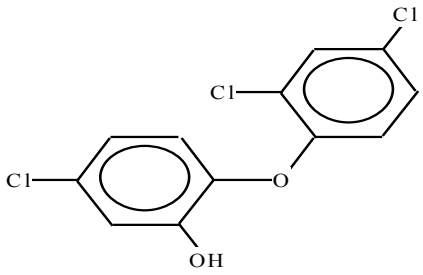

Figure 1.3 – Phenol, 5-chloro-2-(2,4-dichlorophenoxy.

Triclosan is primarily found in wastewater effluent, as well as in sewage sludge (Boyd et al., 2003; Hua et al., 2005). Studies on the removal of TRI through wastewater treatment facilities have shown little to no efficiency from influent to effluent stage (Carmona et al., 2014). The greatest reduction of Triclosan, however, is typically seen with lagoon treatment, as opposed to conventional activated sludge (Lishman et al., 2006). DWTPs have been shown to reduce Triclosan concentrations during treatment process (3.3 to 1.4 ng/L) (Padhye et al., 2014). To the best of our knowledge, there have been only two studies conducted thus far on the presence of Triclosan in finished drinking water in Ontario, and neither of them detected concentrations of TRI above their detection limits (Boyd et al., 2003; Servos et al., 2007). Being such a prevalent and toxic contaminant, water quality guidelines have set the limit to 0.115 μg/L (Hull et al., 2015). However, this is not an enforced regulation, but rather, a suggested safety limit.

Unfortunately, recent literature has measured Triclosan concentrations as high as 600 ng/L outside of wastewater effluent discharge locations, specifically in Lake Ontario (Csiszar et al., 2011). In addition, TRI was present in various effluent and surface water samples of Canadian shorelines of the Upper Detroit River (Hua et al., 2005). Due to the high prevalence and physiochemical properties of this contaminant, it is being found in various organisms, such as, rainbow trout, wild fish, eelpout, and perch (Hua et al., 2005).

Microalgae is presently thought to be extremely sensitive to the presence of Triclosan, as it appears that environmentally realistic concentrations of this compound in aquatic systems has been shown to result in negative impacts to algal biofilms and community assemblages (Reiss et al., 2002; Ricart et al., 2010; Tamura et al., 2012). Significant changes in algal and cyanobacterial community assemblages are occurring with exposure to TRI, both in laboratory

and field experiments, where cyanobacteria appear to be more resistant to the toxicological effects of TRI than that of eukaryotic algae (Drury et al., 2013; Lawrence et al., 2015). A recent study has suggested that filamentous algae may be able to make for an excellent indicator for estimating the bioaccumulative potential of Triclosan (Coogan et al., 2007).

1.1.8 Common Indicators of PPCP Contamination

One of the simplest methods of confirming the presence/absence of a contaminant, would be to simply conduct chemical monitoring. There are two commonly used methods for collecting these samples, including instantaneous water samples and time-weighted average samples. Both methods have proven to be a useful and accurate tools for the estimation of this compound in the water system (Li et al., 2010). The most commonly used methods for analyzing the PPCPs in the water samples are the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS), High Performance Liquid Chromatography (HPLC) and/or bioassays (Z. Liu et al., 2009). As such, various bio-indicators have been looked at as an alternative method for predicting the presence/absence of a contaminant.

A variety of invertebrates and fishes have been proven to be quite useful indicator of assessment of water pollution, particularly rainbow trout (fish), daphnia (zooplankton), and green microalgae (Dodds et al., 2010; US EPA, 2016). The sensitivity to specific degrees of pollution and environmental gradients are the characteristics that makes a particular species as a good bioindicator (Dodds et al., 2010). While assessing aquatic toxicity, calculating RQ values for a given contaminant against daphnia is considered as a useful indicator (ChemSafetyPro, 2015).

Using bioindicators is often a cost-effective alternative to chemical monitoring of ecosystem health. Algae, although typically microscopic in size, their role in maintaining the foundation of our aquatic food chain is vital. As such, in some scenarios they are the first to be affected by a particular contaminant, and in turn can cause major changes, sometimes detrimental. Microalgae, are extremely sensitive to changes in the environment, and these changes can be seen in just a short period of time compared to other organisms that require more time for expressing the effects. For these reasons, microalgae make an excellent biological indicator of water quality. It has been shown that parameters such as growth inhibition, lipid content, pigment content, inhibition of photosynthesis, and metabolic processes are some of the

algal characteristics that could predict the health of an aquatic ecosystem (Julius et al., 2007; Moro et al., 2014). Microalgae can be divided into several groups, some of the most prevalent and abundant groups in our freshwater systems being Bacillariophyceae (diatoms), Chlorophyceae (green algae), Chrysophyceae (golden algae), and cyanobacteria (blue-green algae). Chlorophyceae is typically studied as a bioindicator due to their prevalence and being the most diverse freshwater algae group (Dodds et al., 2010). However, there are most certainly benefits to studying some of the other groups of algae, particularly Bacillariophyceae.

1.1.9 Diatoms as a Bioindicator

Diatoms, belong to the class, Bacillariophyceae, and are one of the major communities of freshwater algae found in both marine and freshwater systems due to their vast abundance and diversity (Wehr et al., 2015). The cell walls of these unicellular algae are made up of silicone dioxide and exist in a wide variety of sizes, shapes, and sculptures (Smol & Stoermer, 2010; Wehr et al., 2015). This unique cellular composition allows for the preservation of their valves in freshwater sediments (Pienitz et al., 1995; Smol & Stoermer, 2010). These algae synthesize their cell walls with less energy than that of the other algal communities (Sigee, 2005). More specifically, diatoms are able to photosynthesize sugars into lipids, constituting them as an excellent food-source in freshwater systems (Julius et al., 2007). Diatoms are typically quite easy to culture in a laboratory setting. Several diatom species have shown narrow tolerance ranges for chemical and physical environmental variables (Bothwell, 1988; Cattaneo et al., 2004; Y. Liu et al., 2010; Morin et al., 2010). For these reasons stated above, this study explores toxicological effects of PPCPs on diatoms.

The European Diatom Database (EDDI) is a commonly referenced web-resource whereby diatoms from Europe, Asia, and Africa are described including their potential to act as ecological indicators (“European Diatom Database,” 2015). Diatoms are used to indicate surface water acidification, eutrophication, climate change, and more (“European Diatom Database,” 2015). This index provides ecologists with a quick, cost-effective method for understanding their aquatic environments. Unfortunately, this list has not yet been updated for North America.

Within North America, there have been several studies conducted in regards to diatoms as an aquatic bioindicator of nutrient enrichment, invasive species introductions, paleolimnological climatic changes, etc. (Bothwell, 1988; Pienitz et al., 1995; Winter et al.,

2011). However, very little work in terms of ecotoxicological investigations have been done on diatoms as an indicator for PPCP contamination. It is known, however, that diatoms may experience changes in chlorophyll-*a* content, undergo morphological changes, and/or experience a change in growth rate due to exposure from PPCPs (Julius et al., 2007). One study, found that exposure to Triclosan, resulted in decreased diatom species diversity and richness (Morin et al., 2010). In another study, the effects of EDCs on a diatom, *Navicula sp.*, indicated an increase in lipid content and high bioaccumulation rates (Y. Liu et al., 2010). There are also studies that reported changes in microalgal composition due to exposure from EDCs; from diatom/green algae dominant community to cyanobacteria dominant community (Bácsi et al., 2016; Drury et al., 2013; Lawrence et al., 2015).

1.1.10 Algal Species for Study

Two diatom species have been chosen for this study: *Asterionella formosa* and *Diatoma tenuis*. These diatoms are widespread and abundant in the freshwater systems of south-central Ontario. *A. formosa* has been long studied for sensitivities to environmental changes including eutrophication, nutrient depletion, calcification, and the introduction of invasive species (Barbiero et al., 2011; Barrow et al., 2014; Sivarajah et al., 2016). Less information pertaining to *Diatoma sp.* as a bioindicator exist, however, both its prevalence in Great Lakes region and robust nature in a laboratory setting is reason enough to explore the toxicological end points of this species.

1.1.10.1 *Asterionella formosa*

The origin of this species name is in Latin, translating to 'beautiful' (M.D. Guiry, 2016a). *A. formosa* is a freshwater species of algae, belonging to the class, Bacillariophycaea (Figure 1.4). The cell length varied from 45 – 70 µm (M.D. Guiry, 2016a). This species can be found in a variety of different freshwater systems across the globe, but particularly in lakes and slow moving rivers (M.D. Guiry, 2016a). This species has been important in nutrient consumption and paleolimnological studies, as it has an incredible ability to store phosphorus and is one of the slowest sinking diatoms of its community (Lotter et al., 1998; Wehr et al., 2015). Several studies suggest, *A. formosa* to be an excellent indicator of eutrophication or hypertrophication, as well an indicator of metal contamination (i.e. aluminium) (Cattaneo et al., 2004; Lotter et al., 1998; Rawson, 1956).

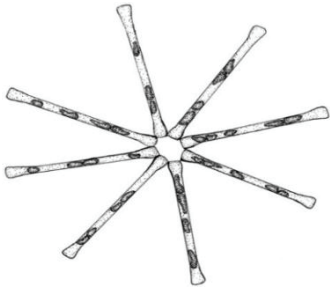

Figure 1.4 - A typical colony formation of *Asterionella formosa* (M.D. Guiry, 2016a).

1.1.10.2 *Diatoma tenuis*

This diatom, *D. tenuis*, is a common species of North American freshwaters however they are found in brackish water ecosystem as well (M.D. Guiry, 2016b). The cell length ranges from 15-60 µm and often form 'zig-zag' colonies (Figure 1.5). This diatom has commonly been used an indicator of eutrophication (Dokulil et al., 1997; Rawson, 1956). Outside of phosphorus enrichment, very little work has been conducted on this diatom.

Figure 1.5 - Filamentous colonies of *Diatoma sp.* (M.D. Guiry, 2016b).

1.2 Rationale and Objectives

1.2.1 Rationale for the Study

A review of the literature suggests that data on the effects of these compounds on primary producers are still lacking. In addition, a gap of knowledge exists in the occurrence of PPCPs in Lake Simcoe, particularly in the nearby areas of WWTPs which discharge effluents into the adjoining water bodies. Examining the sensitivities of the diatom community to three PPCPs (17-β Estradiol, Ibuprofen, and Triclosan) would prove useful to develop a fast and cost-effective method for assessing the extent of PPCP contamination in our inland waters. The three

compounds were chosen based on their prevalence as reported in the previous studies on Lake Simcoe and Great Lakes. Moreover, as mentioned before the diatom species, *A. formosa* and *D. tenuis*, are prevalent in Lake Simcoe and Great Lakes thus helping us to have a good estimate of the impacts that these compounds will cause on the primary producers.

1.2.2 Study Objectives

This study aims to address the usefulness of these diatom species as indicator of PPCP contamination in Lake Simcoe. The laboratory component will explore the toxicological impacts of Ibuprofen, 17-β Estradiol, and Triclosan on two diatom species. Individual and a combination of these PPCPs exposure will be examined to answer two research question: 1) Are these microalgae more sensitive to PPCP exposure than species belonging to other algal communities, and; 2) Do these PPCPs cause compounding effects on these species when exposed in combination and whether the combination effect is more severe than individual effects on these diatoms? Furthermore, a field component will help determine the occurrence of three PPCPs (Ibuprofen, Estrone, and Triclosan) in the nearby areas of the three WWTPs that discharge their effluents into a river/creek that feeds into Lake Simcoe. PPCP contamination will be studied at the point of effluent discharge, as well as, at the point of confluence (of the creek) with the lake. In addition, several water and algal parameters will be monitored at these sites. This component is carried out to answer the final research question: 1) Is diatom composition influenced by the presence of any or all three PPCPs in this area? Thus, the null hypothesis of this study is that a combination of PPCPs will not result in a more severe impact on the diatom species, *A. formosa* and *D. tenuis*, than individual exposure. Secondly, diatom composition will not be significantly affected by the presence of these compounds and therefore diatoms can be used as indicators of these compounds in the water.

2. Individual and Combined Toxicity Testing of Three PPCPs on Diatoms – A Laboratory Study

2.1 Introduction

Pharmaceutical and Personal Care Products (PPCPs) in our freshwater systems has become an emergent issue in Lake Simcoe, with the potential of affecting many of the inhabiting organisms (Metcalfe, 2014; Wray et al., 2014). Primary producers, such as the micro-algae play an important role in maintaining trophic stability (Smol & Stoermer, 2010). They often exhibit extreme sensitivities to changes in their environment and as such, are often used as indicators of aquatic health (Julius et al., 2007; Moro et al., 2014; Smol & Stoermer, 2010). The main objective of this study is to explore the toxicological effects of three PPCPs (17-β Estradiol, Ibuprofen, and Triclosan) on the growth rate of two diatom species, common in the Lake Simcoe watershed. These toxicological values will be compared to other studies, where the effects of these compounds were tested on either microgreen algae, cyanobacteria, and/or algal composition. It is hypothesized that these diatoms will be more sensitive to these PPCPs than algae from other communities and as such, would make diatoms an excellent candidate as an indicator for pharmaceutical contamination in Lake Simcoe. In addition, these two diatoms will be exposed to a combination of these three compounds in efforts of exploring synergistic effects. It is quite common for these compounds to appear in wastewater effluents simultaneously and as such, should be examined in a similar manner. It is suspected that mixture toxicity will result in compounding effects as seen in studies that used similar methods to test other PPCPs (Cleuvers, 2004; Geiger, 2014; Ginebreda et al., 2014).

2.2 Methodology

The parent cultures of the diatom species *Asterionella formosa* (ID #692) and *Diatoma tenuis* (ID #62) were collected from Lake Erie (2009) and Lake Ontario (1984), respectively, and were purchased from the Canadian Phycological Culture Centre (CPCC), University of Waterloo, ON, Canada. Both cultures were reported as having bacterial contamination. Cultures were covered with a cotton plug and bio-shield in efforts of reducing further viral and/or bacterial contamination. All cultures were kept in an environmental chamber with a temperature of 20ºC ±0.2, humidity of 15% ±5, and light intensity of 60 µmol◦s/m ±10. The cultures were thoroughly mixed once daily. The source of light used in the chamber was fluorescent bulbs, and

ran on a 12:12 photoperiod. The cultures were grown in a modified CHU-10 media specially developed for the growth of freshwater diatom species in the Great Lakes (Stein, 1973). All glassware used in the study were properly washed (traditional-, acid-, organic-) and sterilized prior to use in order to avoid bacterial, nutrient, or chemical contaminations. All samples containing algal species that were no longer required for the study were destroyed and discarded in an environmentally safe manner.

The glassware used for this study was first washed with a 10% solution of hydrochloric acid, followed by an organic rinse with 90% acetone before sterilization in an autoclave. Only deionized (DI) water was used for rinsing, making stock solutions, and media preparation. All media were sterilized in glassware capped with a non-absorbent cotton plug and bio-shield cover in an autoclave prior to inoculation. The pH meter was calibrated on a daily basis by using standard solutions.

2.2.1 PPCP Solutions Preparation

2.2.1.1 17-β Estradiol

The 17-β estradiol was purchased from Sigma-Aldrich. 50 mg of this chemical was weighed out using the analytical balance using a sterile spatula and non-absorbent weighing paper. The powder was carefully transferred into a 500 mL volumetric flask with 50 mL of DI water. 500 µL of 1 N NaOH solution was added to the flask, followed by thorough mixing. Once the powder was completely dissolved, the flask was topped up with DI water.

2.2.1.2 Ibuprofen

The ibuprofen used throughout this study was taken from gel capsules purchased at a local retail store. The capsules were perforated with a sterile blade and weighed using an analytical balance. It was then transferred into a 1 L volumetric flask with 100 mL of DI water. The solution was titrated with 1 N NaOH (~ 0.5 to 2 mL) until transparent. The flask was then topped up with DI water.

2.2.1.3 Triclosan

A 500 mL volumetric flask is filled with 50 mL of DI water. The entire flask is weighed on the analytical balance, prior to the addition of 65 mg of triclosan. The flask is mixed

thoroughly with 200 µL of 1 N NaOH solution. Once the powder is fully dissolved, the flask is topped with DI water.

2.2.2 Culturing Techniques

The diatom cultures were distributed in 15 mL test tubes. These cultures were sub cultured several times into larger batches until reaching a total volume of 250 mL. All flasks were labelled with media type, diatom strain, date of inoculation, and date of confirmed isolation. This sub-culturing technique was completed inside a biosafety hood. Parent culture was grown to a minimum density of 4 x 10^4 cells/mL and not exceeding 0.5 mg/L in biomass. The cotton plug of both the parent culture and the recipient was removed and set aside. The parent culture was mixed thoroughly by a swirling motion for at least 30 seconds. A 10 mL serological pipette was used to remove 1 mL from the flask containing the parent culture, and swiftly transferred to the appropriately labelled flask containing sterile media. The cotton plugs were replaced and securely fastened with rubber bands. Both flasks were mixed thoroughly and then returned into the environmental chamber or set aside for further analytical measurements

2.2.3 Quantitative Measurements

2.2.3.1 Growth Inhibition Test

The diatom cultures were studied using a batch culturing technique, where all phases of growth curve would be observed. The effects of exposure to three PPCPs was assessed by following Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test as described by the Organization for Economic Co-operation and Development (OECD, 2011). The test organisms were exposed to a minimum of five concentrations of each PPCP in replicates of at least three ($n \geq 18$ per one PPCP). The concentrations used in the experiment were chosen based on previous literature whereby the substance has been tested on other species of algae, typically on Chlorophycaea (Geiger, 2014). Additional concentrations were chosen based on preliminary results, whereby a range from 0 to 100% growth inhibition would be determined.

2.2.3.2 Algal Biomass

Algal biomass is used to determine the growth of the cultured algae, however, due to the difficulty in extracting large volumes of sample to obtain an accurate reading of biomass, a surrogate measurement, algal density, was made. A linear relationship between cell counts and algal biomass was determined for each diatom.

For obtaining algal counts, a hemocytometer method was used. A small aliquot (~ 500 µL) is taken from each sample and placed under the cover slip of the hemocytometer with a sterile pipette, using a Bunsen burner to ensure aseptic condition. A minimum of 100 cells were counted with each sample to maintain a statistically significant population count. Algal biomass was determined by pouring the entire sample over a 1 µm porous glass-fiber filter paper using a Büchner funnel and vacuum. The filter papers were dried in a thermo-regulated chamber at 40ºC for 24 to 48 hours.

After determining the relationship between algal density and algal biomass for each diatom, individual logistic growth curves in presence and absence of toxicological compounds were explored. The algal density measurements were completed every one to three days for the duration of each individual growth curve (~ 20 days), and the following calculation (see Equation 2) was used to calculate the population density per ml.

$$\text{Total cells/ml} = \text{total cells counted} \times \frac{dilution\ factor}{\#\ of\ squares\ in\ hemocytometer} \times 10{,}000 \text{ cells} \quad (2)$$

The population densities would then be plotted on a graph against time, in order to produce growth curve. The growth rate would then be defined from this relationship between cell counts and time (see Equation 3) [4](Dauta et al., 1990). Where, μ is the population density measured in units of organism per day, t is the time measured in units of days, and N_o and N_t represent the population density pre- and post- incubation.

$$\mu = t^{-1} \ln(N_t/N_o) \qquad \text{unit } (day^{-1}) \quad (3)$$

2.2.3.3 Optical Density

The optical density for the diatom cultures was measured three times throughout each experiment: day 1, mid-experiment, and the final day. For obtaining these measurements, 2 mL was taken from each sample and transferred into a clean 3 mL glass cuvette. This cuvette was placed in the spectrophotometer to measure the absorbance at 680 nm (Butterwick et al., 1982).

2.2.3.4 pH Measurements

The pH of the culture was measured at the beginning and at the end of each experiment by using a Hach hydrolab (±0.01). Each treatment group contained an extra sample in which the pH measurements were made. This was done in a separate flask was to avoid contamination.

Any deviations in pH that exceeded 1.5 units during the test, were not accepted in the study as this change would have the potential to significantly affect the growth of the diatom culture.

2.2.4 Statistical Analyses

2.2.4.1 Concentration Response Curves

A logistic growth curve was drawn for each diatom species under varying concentrations of PPCP(s). This was done by plotting cell counts against time (days). Average specific growth rate was calculated for each single sample of controls and treatments by calculating the logarithmic increase in the cell counts (see Equation 4). Where, $\mu_{i\text{-}j}$ is the average specific growth rate from time i to j, X_i is the cell counts at time i, and X_j is the cell counts at time j.

$$\mu_{i\text{-}j} = \frac{\ln Xj - \ln Xi}{tj - ti} \ (\text{day}^{-1}) \qquad (4)$$

A Randomized Blocked Analysis of Variance (ANOVA) was used to identify at what concentrations the growth curve started to significantly vary with respect to the control and amongst varying PPCP concentrations. Thus, the hypothesis tested during this study was:

> H_0: Exposure to PPCP concentrations has no significant effect on the growth rate of a diatom species

The appropriate tests used to validate this analysis also included a test of homogeneity of variances and a test of normality in the data. This was done by using Bartlett test and Anderson Darling test, respectively. A Tukey post-hoc test was used to further identify significant difference between pairwise concentrations of PPCP on the growth rates ($\alpha = 0.05, 0.10$).

2.2.4.2 Growth Inhibition Responses

Once a significant effect of PPCP concentrations on mean growth curves was detected, a regression analysis using these data was conducted between PPCP concentrations ($\log_{10}$) and percent inhibition of growth rate (see Equation 5). The established linear relationship between the two variables could be used for determining the predictive toxicological values. Where, $\%I_r$ is the percent inhibition in average specific growth rate, μc is the mean value for average specific growth rate μ in the control group, and μt is the average specific growth rate for the treatments.

$$\%I_r = \frac{\mu c - \mu t}{\mu c} \times 100 \qquad (5)$$

2.3 Results

2.3.1 Specific Growth Correlations

The two diatom species, *Asterionella formosa* and *Diatoma tenuis,* were initially examined for their specific growth curves (Figures 2.1 and 2.2). Both species took five days to reach the exponential growth phase. Maximum carrying capacity was reached for *A. formosa* and *D. tenuis* by day 13 and 10, respectively. The carrying capacity of *A. formosa* and *D. tenuis* was approximately 560,000 and 160,000 cells/mL, respectively.

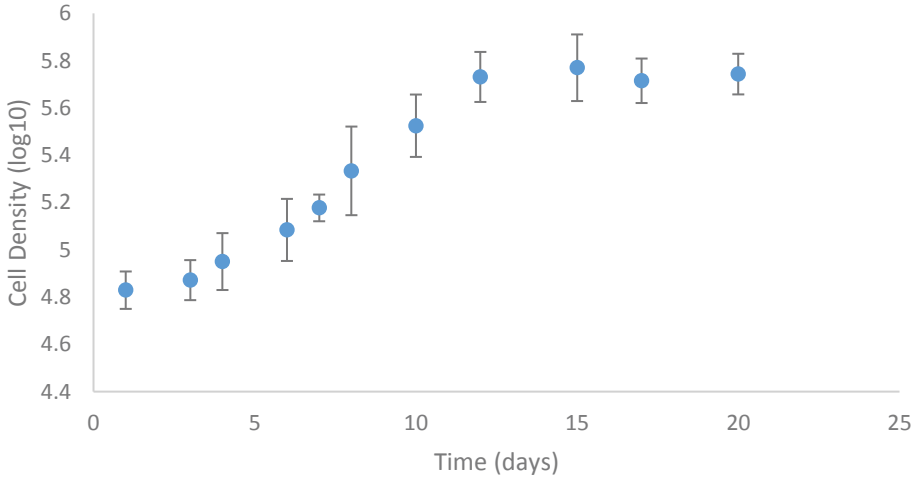

Figure 2.1 - Logistic growth curve of *Asterionella formosa.*

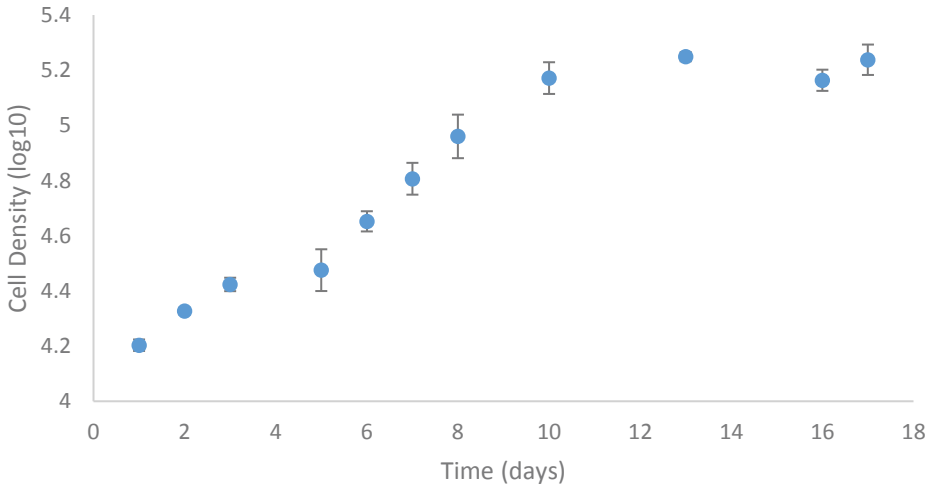

Figure 2.2 - Logistic growth curve of *Diatoma tenuis.*

The relationship between algal biomass, cell counts, and optical density was also examined for both species. The suggested wavelength used for measuring optical density of diatoms was 680 nm (Butterwick et al., 1982). A wavelength scan of both species was conducted to confirm that a peak wavelength occurred at this precise point. As illustrated below, these three variables are closely related and thus, it would be acceptable to use optical density and cell density as surrogate parameters for algal biomass under these controlled conditions (Figures 2.3 and 2.4).

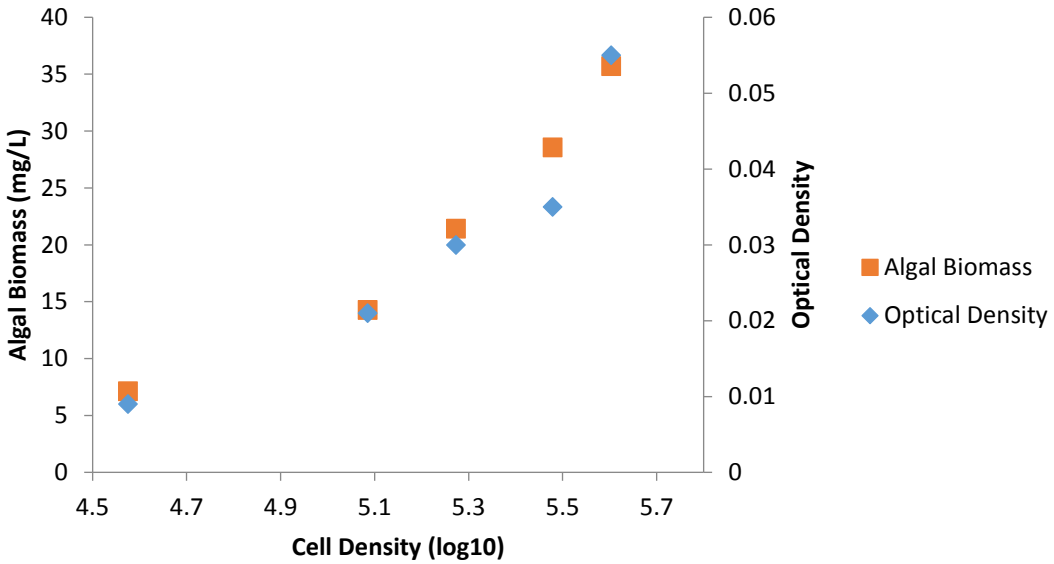

Figure 2.3 - Specific growth relationship between algal biomass, cell counts, and optical density for *Asterionella formosa*.

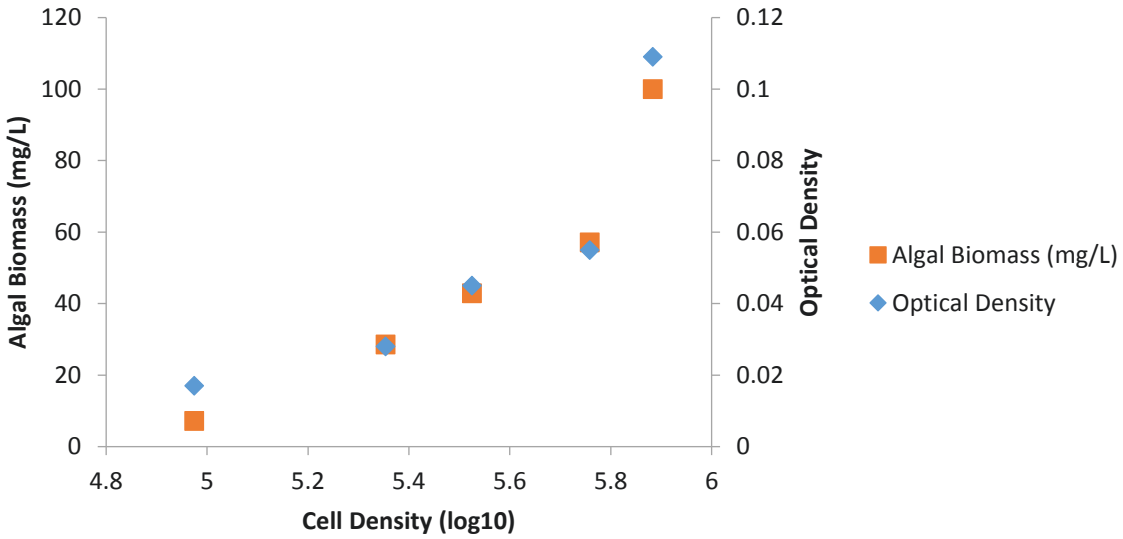

Figure 2.4 - Specific growth relationship between algal biomass, cell counts, and optical density for *Diatom tenuis*.

2.3.2 Single Toxicity Testing

2.3.2.1 Concentration Response Curves

Each toxicological exposure test included a control and a minimum of four different concentrations. Most experiments were repeated using at least two additional concentrations in order to assure enough points that would fit between the range of 0 to 100% growth inhibition. The concentrations (mg/L) tested for *A. formosa* were as follows: Ibuprofen (0.4, 0.8, 1.6, 3.2, 5, 8, 10, 12, 20 and 40), 17-β Estradiol (0.4, 0.8, 1.6, 3.2, 5, 8 and 12), and Triclosan (0.01, 0.02, 0.05, 0.06 and 0.10). The concentrations (mg/L) tested for *D. tenuis* were as follows: Ibuprofen (5, 10, 25, 50, 100, 200 and 400), 17-β Estradiol (0.4, 0.8, 1.6, 3.2, 5 and 10), and Triclosan (0.01, 0.02, 0.04, 0.08, 1.0, 1.6 and 3.2). Dose-response curves were evident in both *A. formosa* and *D. tenuis* for all PPCPs in study based on the results of Randomized Blocked ANOVAs. Thus, the null hypothesis that exposure to PPCP concentrations would have no significant effect on the growth rate of a diatom species is rejected.

2.3.2.1.1 Asterionella formosa

From the data, the percentage inhibition of growth versus individual PPCP concentrations for *A. formosa* was established. Not all PPCP concentrations tested were used in the determination of concentration response values, as not all of them proved statistical significance

between one concentration to the next. As a result, all studies were completed multiple times for testing a series of different concentrations in each trial. A Randomized Blocked ANOVA was used to confirm which concentrations of PPCP caused a significant effect on the growth rate of this diatom species.

The first trial of Ibuprofen toxicity on *A. formosa* included four different concentrations ranging between 0 and 3.2 mg/L (Figure 2.5). None of these concentrations caused any significant effect on the overall growth rate of the species ($F_{4,39} = 1.974$, $p = 0.118$, $t = 14$). As a result, additional tests were performed with higher concentrations.

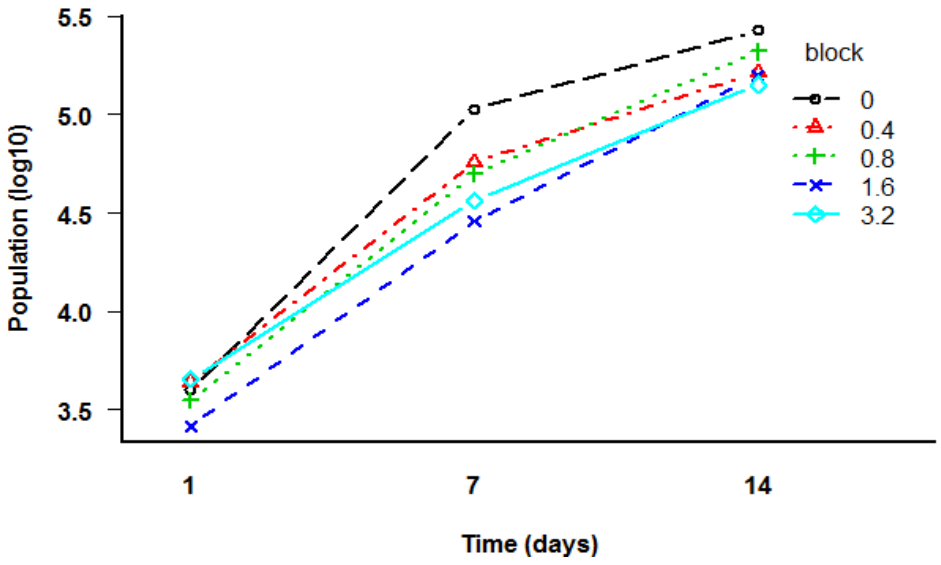

Figure 2.5 – An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Ibuprofen over the course of the first trial.

The second trial tested three concentrations ranging between 5 and 12 mg/L, whereby a significant effect on the growth rates of the species was found ($F_{3,31} = 3.443$, $p = 0.029$, $t = 8$) (Figure 2.6). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.915$ and $p = 0.250$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 5, 0 - 8, and 0 - 12 mg/L ($\alpha = 0.10$).

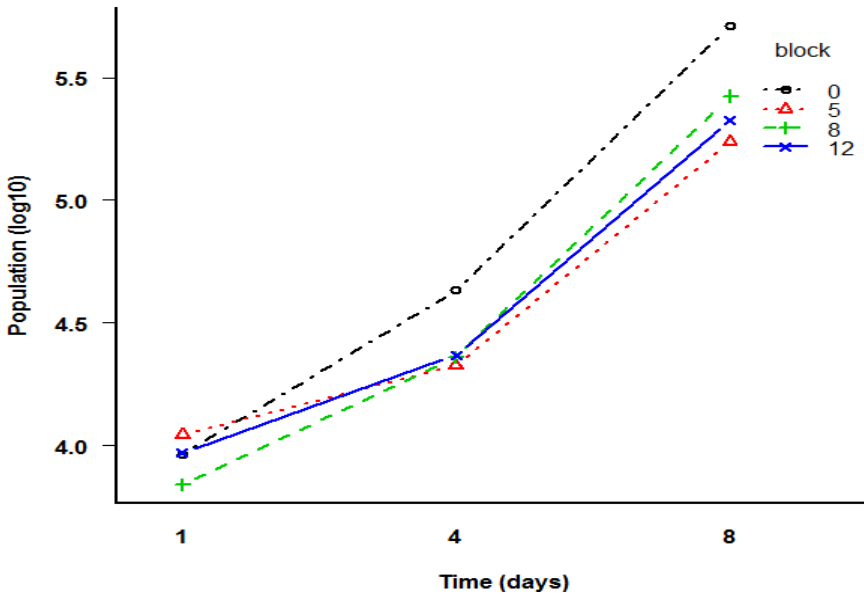

Figure 2.6 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Ibuprofen over the course of the second trial.

The third and final trial tested three more concentrations (10, 20, and 40 mg/L), whereby a significant effect on the growth rates of the species was found ($F_{3,31} = 17.04$, $p = 1.01 \times 10^{-6}$, $t = 11$) (Figure 2.7). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.083$ and $p = 0.611$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 20, 0 - 40, 10 - 40, and 20 - 40 mg/L ($\alpha = 0.05$).

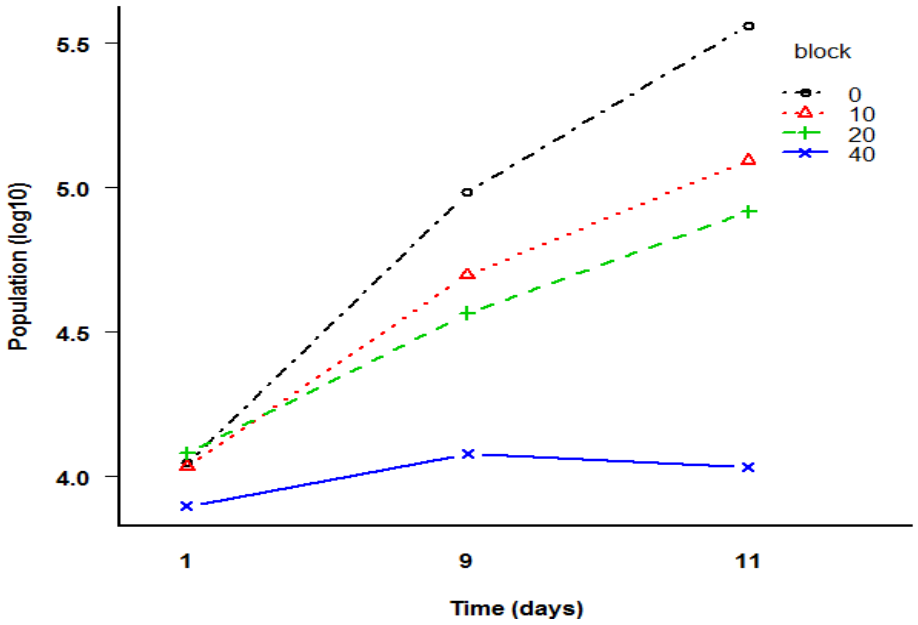

Figure 2.7 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Ibuprofen over the course of the third trial.

The concentrations of Ibuprofen that caused a significant effect on the growth rate of *A. formosa* were 5, 8, 10, 12, 20, and 40 mg/L. The specific growth rates of the samples at the specified concentrations were compared to that of the control samples to establish percentage of growth inhibition (Figure 2.8).

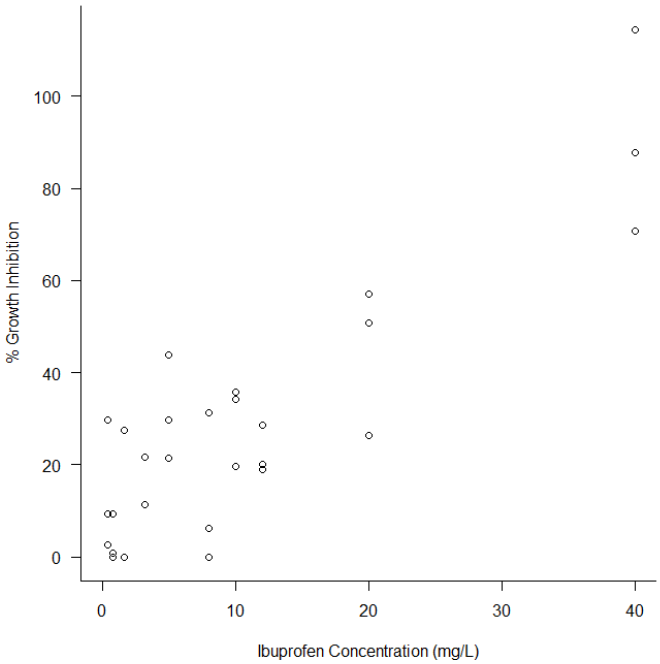

Figure 2.8 – A scatter plot illustration of percentage of growth inhibition with varying Ibuprofen concentrations for *Asterionella formosa*.

The first trial of 17-β Estradiol toxicity on *A. formosa* included four different concentrations ranging between 0 and 3.2 mg/L (Figure 2.9). None of these concentrations caused any significant effect on the overall growth rate of the species ($F_{4,24} = 1.691$, $p = 0.185$, t=7). As a result, additional tests were performed with higher concentrations.

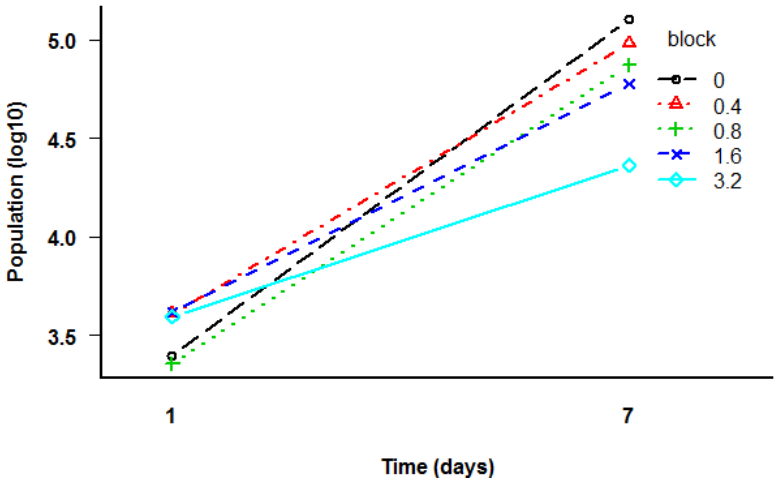

Figure 2.9 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with 17-β Estradiol over the course of the first trial.

The second trial tested three concentrations ranging between 5 and 12 mg/L, whereby a significant effect on the growth rates of the species was found ($F_{3,31} = 10.35$, $p = 7.05 \times 10^{-5}$, t = 8) (Figure 2.10). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.026$ and $p = 0.523$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 5, 0 - 8, 0 - 12 mg/L ($\alpha = 0.05$).

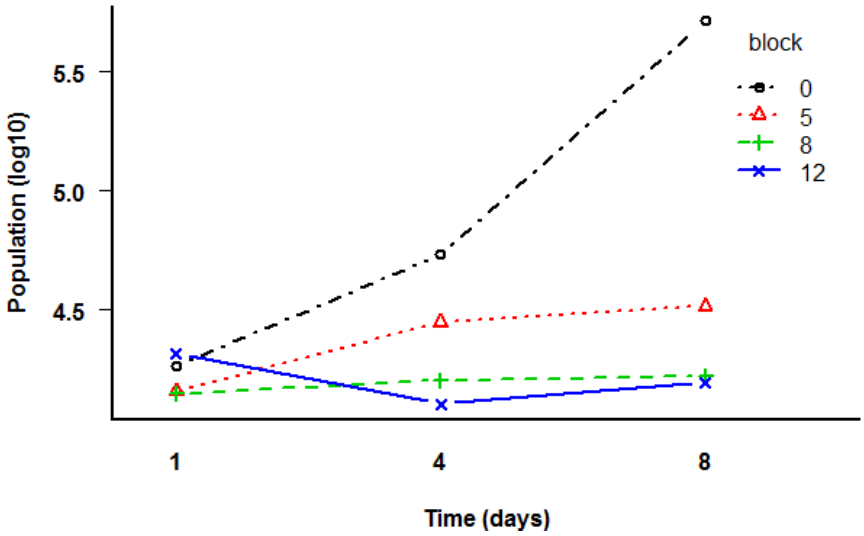

Figure 2.10 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with 17-β Estradiol over the course of the second trial.

The concentrations of 17-β Estradiol that caused a significant effect on the growth rate of *A. formosa* were 5, 8, and 12 mg/L. The specific growth rates of the samples at the specified concentrations were compared to that of the control samples to establish percentage of growth inhibition (Figure 2.11).

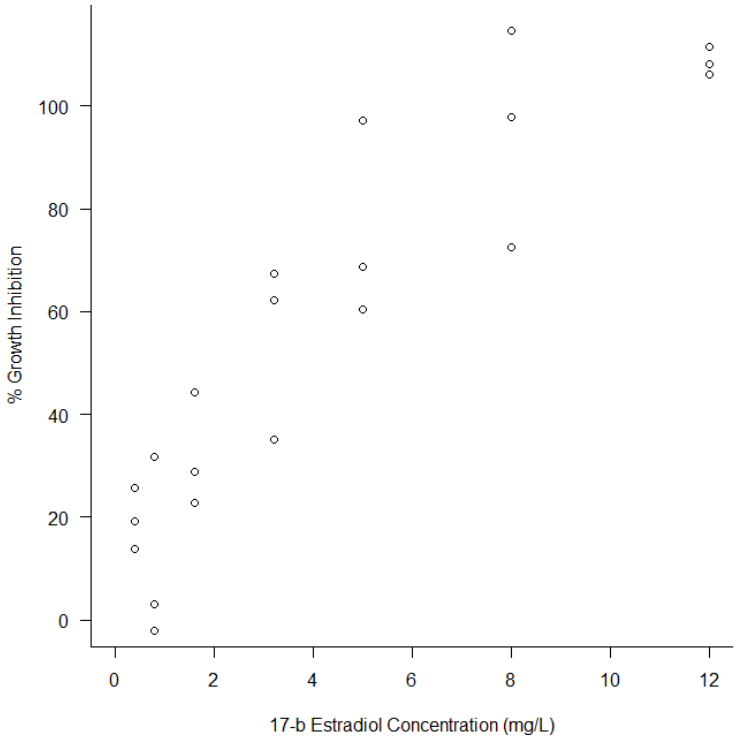

Figure 2.11 - A scatter plot illustration of percentage of growth inhibition with varying 17-β Estradiol concentrations for *Asterionella formosa.*

The first trial of Triclosan toxicity on *A. formosa* included three different concentrations ranging between 0 and 0.1 mg/L, whereby a significant effect on the growth rates of the species was found ($F_{3,139} = 6.272$, $p = 0.001$, $t = 12$) (Figure 2.12). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.042$ and $p = 0.833$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 0.06 mg/L, and 0 - 0.1 mg/L ($\alpha = 0.05$).

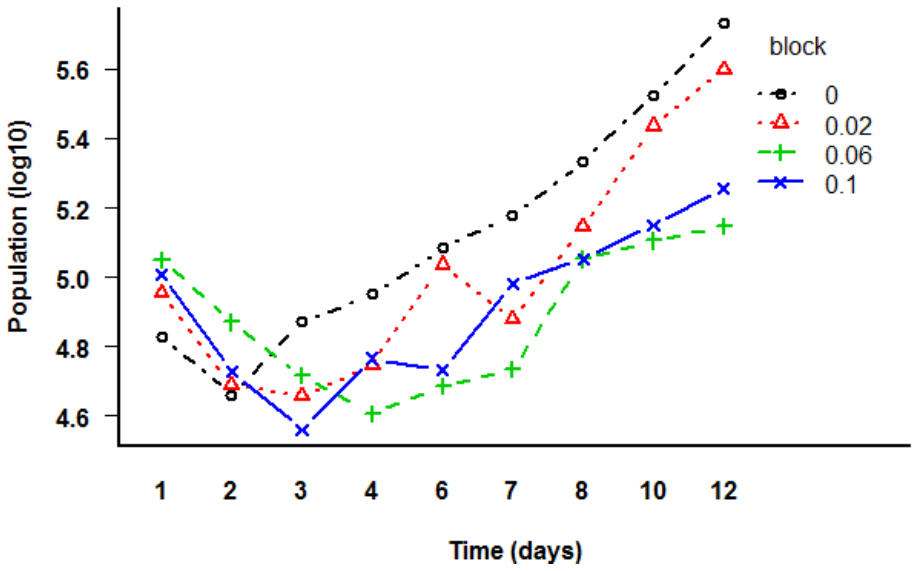

Figure 2.12 - An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Triclosan over the course of the first trial.

The second trial tested an additional two concentrations (0.01 and 0.05 mg/L), whereby a significant effect on the growth rates of the species was found ($F_{2,14} = 2.9$, $p = 0.08$, $t = 8$) (Figure 2.13). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.230$ and $p = 0.249$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 0.05 mg/L ($\alpha = 0.10$).

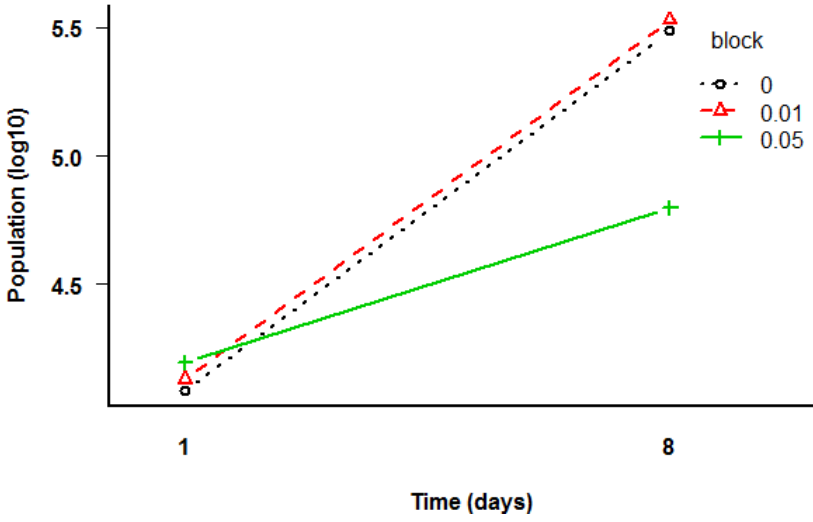

Figure 2.13 – An illustration of a Blocked ANOVA depicting changes in cell density of *Asterionella formosa* with Triclosan over the course of the second trial.

The concentrations of Triclosan that caused a significant effect on the growth rate of *A. formosa* were 0.01, 0.05, 0.06, and 0.1 mg/L. The growth rates of the samples at the specified concentrations were compared to that of the control samples to establish percentage of growth inhibition (Figure 2.14).

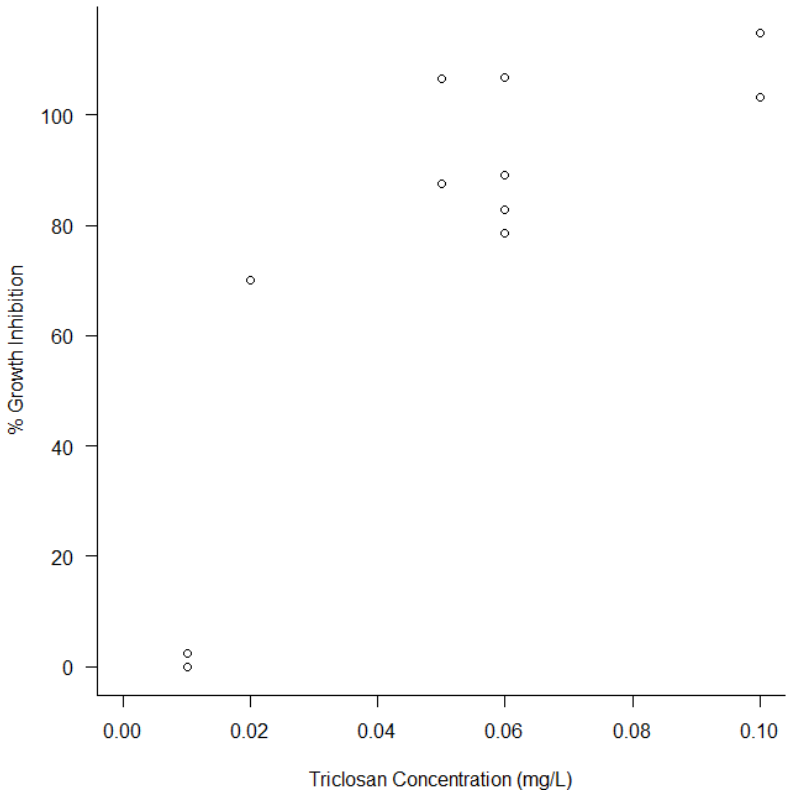

Figure 2.14 - A scatter plot illustration of percentage of growth inhibition with varying Triclosan concentrations for *Asterionella formosa*.

2.3.2.1.2 *Diatoma tenuis*

From the data, the percentage inhibition of growth versus individual PPCP concentrations for *Diatoma tenuis* was established. Similar to the experiments of *A. formosa*, not all PPCP concentrations tested were used in the determination of concentration response values, as not all of them proved statistical significance between one concentration to the next. As a result, all studies were completed multiple times testing for a series of different concentrations in each trial. A Randomized Blocked ANOVA was used to confirm which concentrations of PPCP caused significant effects on the growth rate of this diatom species.

The first trial of Ibuprofen toxicity on *D. tenuis* included five different concentrations ranging between 0 and 400 mg/L, whereby a significant effect on the growth rates of the species was found ($F_{5,108} = 25.71$, $p = 2 \times 10^{-16}$, $t = 10$) (Figure 2.15). The assumption of homogeneity of variance was marginally violated in the distribution of the data, however, normality was not ($p = 0.0002$ and $p = 0.116$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 25 mg/L ($\alpha = 0.05$).

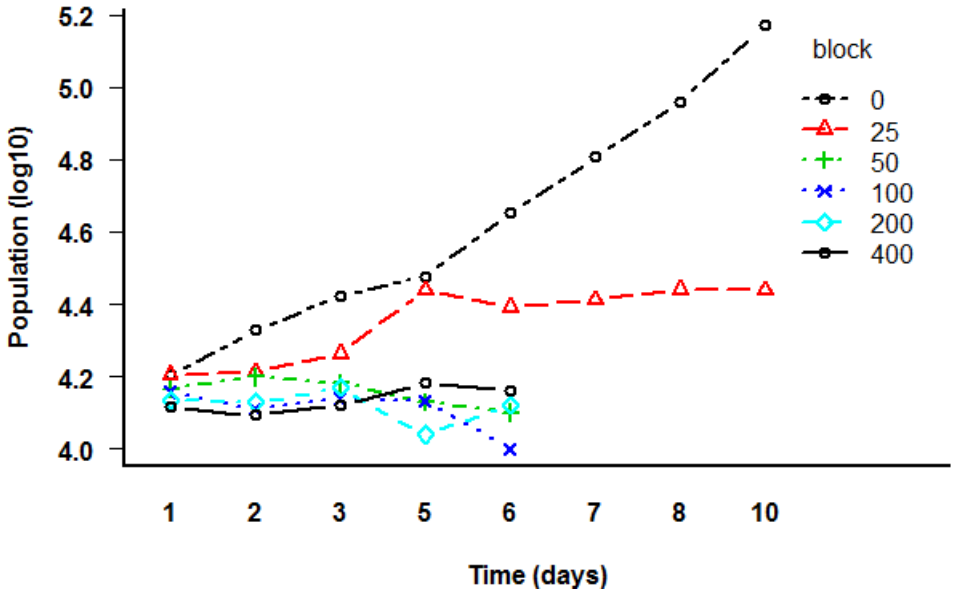

Figure 2.15 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Ibuprofen over the course of the first trial.

The second trial tested for an additional two concentrations (5 and 10 mg/L), whereby a significant effect on the growth rates of the species was found ($F_{2,28} = 9.765$, $p = 0.001$, $t = 14$) (Figure 2.16). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.216$ and $p = 0.797$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 – 10 mg/L ($\alpha = 0.05$).

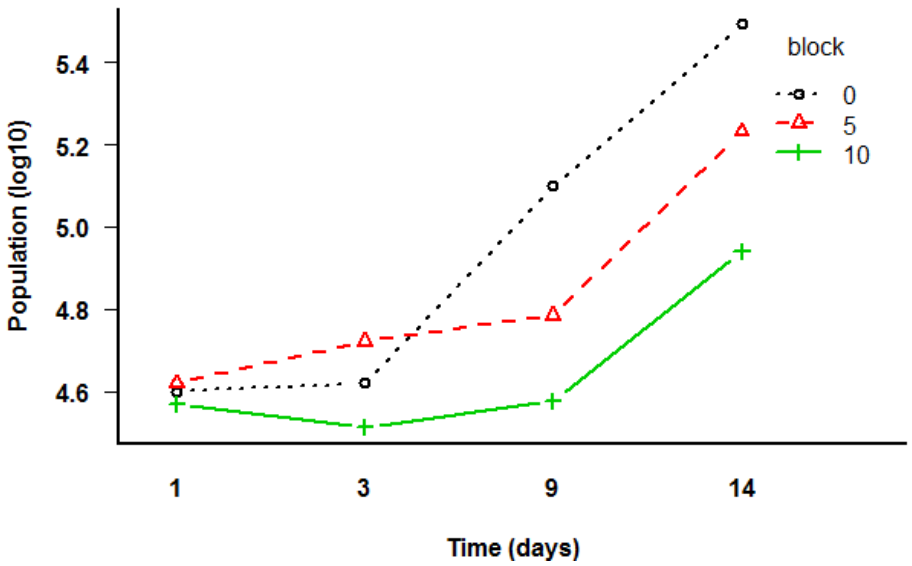

Figure 2.16 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Ibuprofen over the course of the second trial.

The concentrations of Ibuprofen that caused a significant effect on the growth rate of *D. tenuis* were 10 and 25 mg/L. The specific growth rates of the samples at the specified concentrations were compared to that of the control samples to establish percentage of growth inhibition (Figure 2.17).

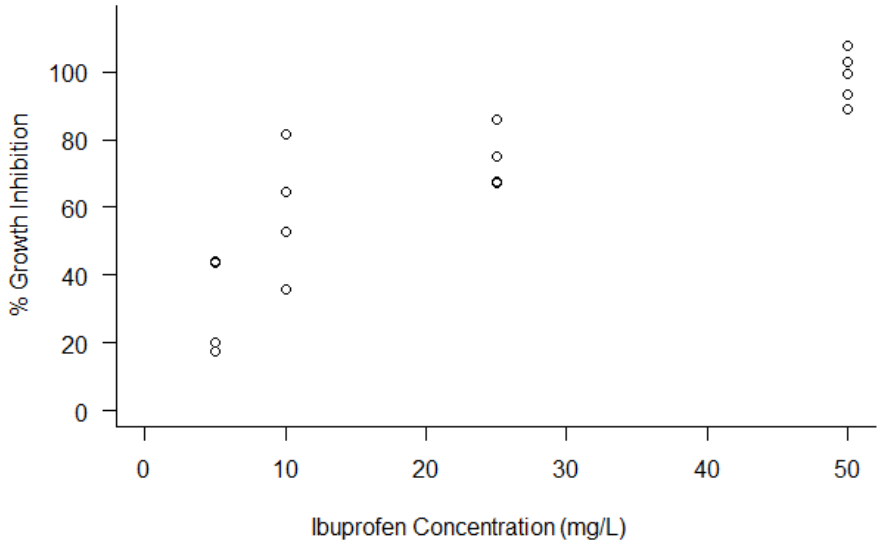

Figure 2.17 - A scatter plot illustration of percentage of growth inhibition with varying Ibuprofen concentrations for *Diatoma tenuis*.

The first trial of 17-β Estradiol toxicity on *D. tenuis* included four different concentrations ranging between 0 and 3.2 mg/L, whereby a significant effect on the growth rates of the species was found ($F_{4,24} = 4.986$, $p = 0.005$, $t = 9$) (Figure 2.18). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.361$ and $p = 0.173$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 3.2 and 0.8 - 3.2 mg/L ($\alpha = 0.05$).

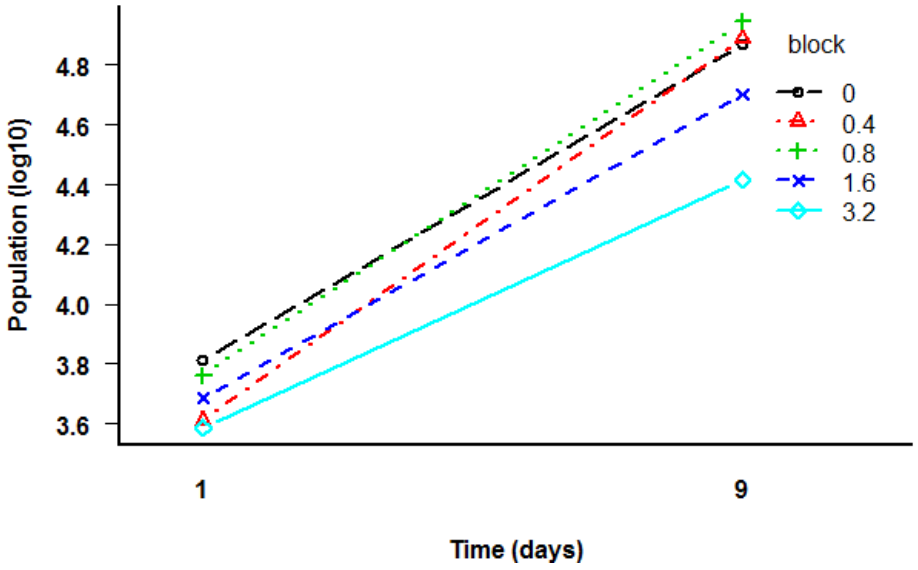

Figure 2.18 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with 17-β Estradiol over the course of the first trial.

The second trial tested for an additional two concentrations (5 and 10 mg/L), whereby a significant effect on the growth rates of the species was found ($F_{2,14} = 6.722$, $p = 0.009$, $t = 15$) (Figure 2.19). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.085$ and $p = 0.609$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 10 and 5 - 10 mg/L ($\alpha = 0.05$).

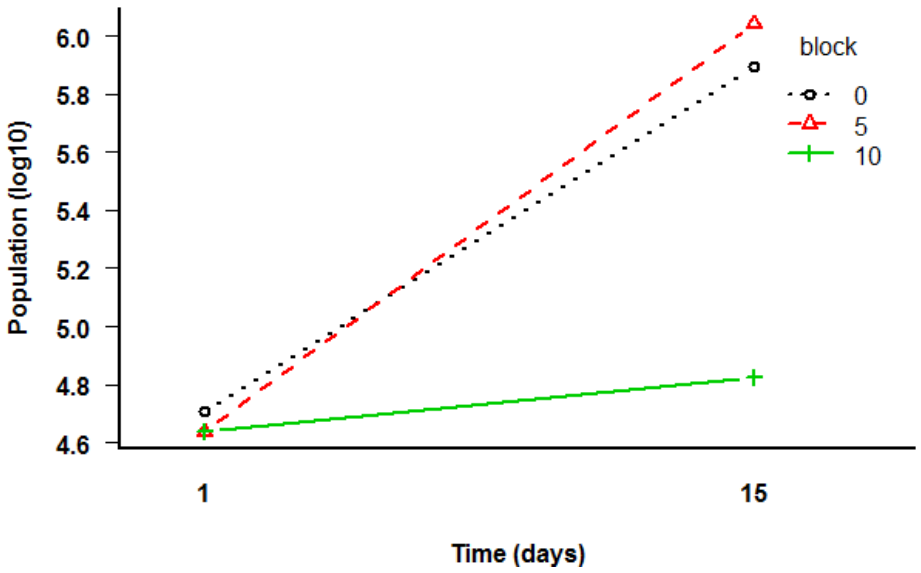

Figure 2.19 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with 17-β Estradiol over the course of the second trial.

The concentrations of 17-β Estradiol that caused a significant effect on the growth rate of *D. tenuis* were 0.8, 3.2, 5, and 10 mg/L. The specific growth rates of the samples at the specified concentrations were compared to that of the control samples to establish percentage of growth inhibition (Figure 2.20).

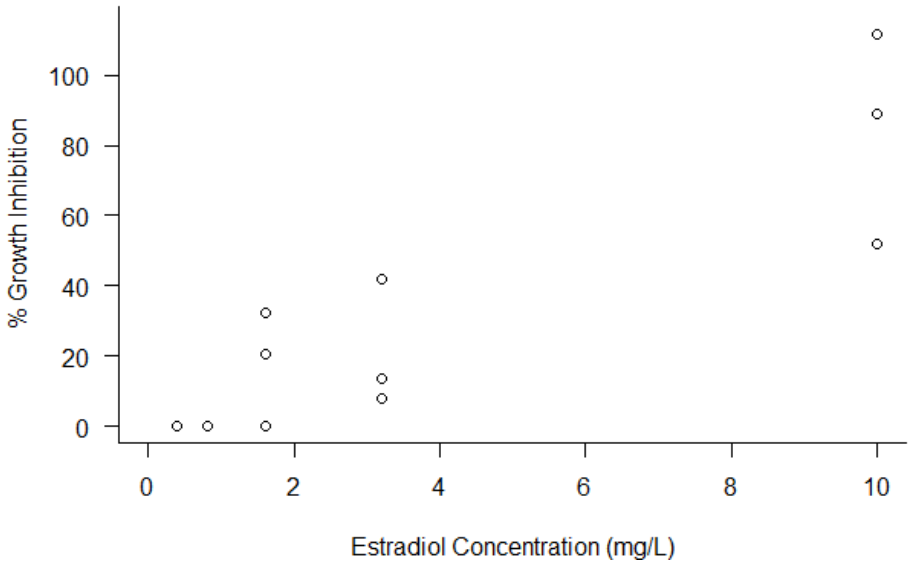

Figure 2.20 - A scatter plot illustration of percentage of growth inhibition with varying 17-β Estradiol concentrations for *Diatoma tenuis*.

The first trial of Triclosan toxicity on *D. tenuis* included four different concentrations ranging between 0 and 0.1 mg/L. None of these concentrations caused any significant effect on the overall growth rate of the species ($F_{4,44} = 1.433$, $p = 0.239$, $t = 15$) (Figure 2.21).

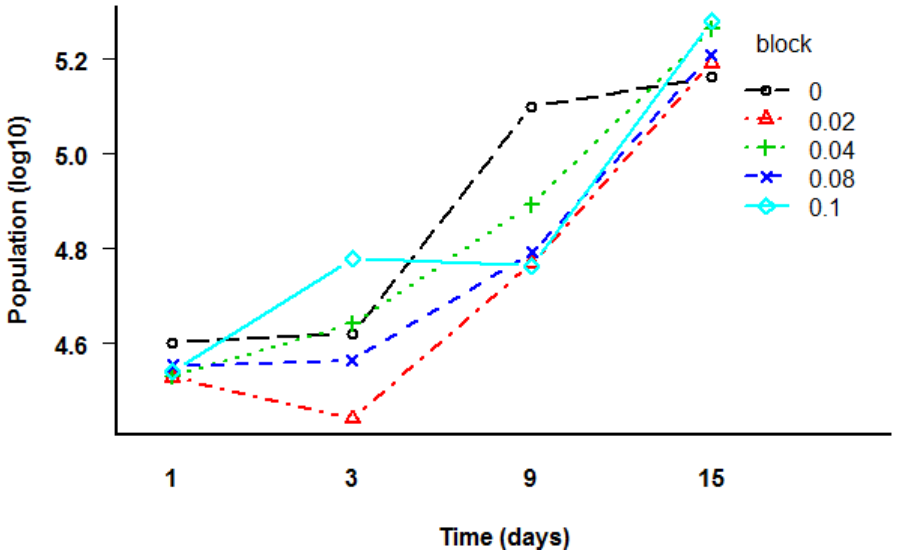

Figure 2.21 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Triclosan over the course of the first trial.

The second trial tested for an additional six concentrations (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/L), whereby a significant effect on the growth rates of the species was found ($F_{6,46} = 5.641$, $p = 0.0001$, $t = 13$) (Figure 2.22). The assumptions of homogeneity of variances and normality were not violated in the distribution of the data ($p = 0.105$ and $p = 0.503$, respectively). The Tukey post-hoc analysis revealed significant differences between concentrations 0 - 0.4, 0 - 0.8, 0 - 1.6, 0.1 - 3.2, and 0.1 - 0.8 mg/L ($\alpha = 0.05$).

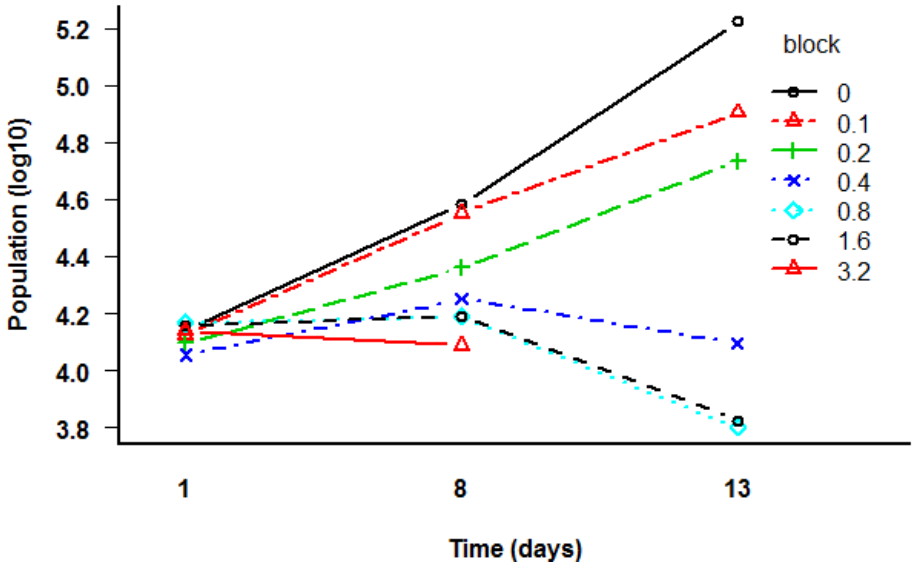

Figure 2.22 - An illustration of a Blocked ANOVA depicting changes in cell density of *Diatoma tenuis* with Triclosan over the course of the second trial.

The concentrations of Triclosan that caused a significant effect on the growth rate of *D. tenuis* were 0.1, 0.4, 0.8, 1.6, and 3.2 mg/L. The specific growth rates of the samples at the specified concentrations were compared to that of the control samples to establish percentage of growth inhibition (Figure 2.23).

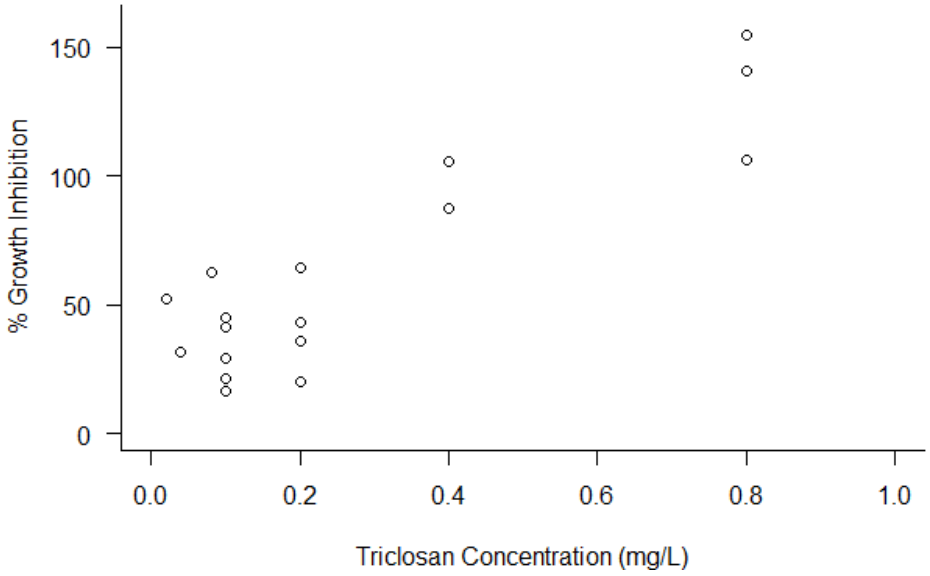

Figure 2.23 - A scatter plot illustration of percentage of growth inhibition with varying Triclosan concentrations for *Diatoma tenuis.*

2.3.2.2 Toxicological Data

Predictive toxicological values for each PPCP in the study were established from the single toxicity testing. These values are based on the effectiveness of a concentration to inhibit the growth of a diatom species, termed as EC_x. Where, *EC* is the effective concentration and *x* is the percentage of growth inhibition exhibited by the concentration. The concentration-response curves were transformed into semi-log plots to obtain a linear relationship between the two variables, log_{10} concentration and percentage inhibition of growth (Figures 2.24 through 2.29). The slope of these linear correlations was then used to calculate predicted toxicological values (Table 2.1).

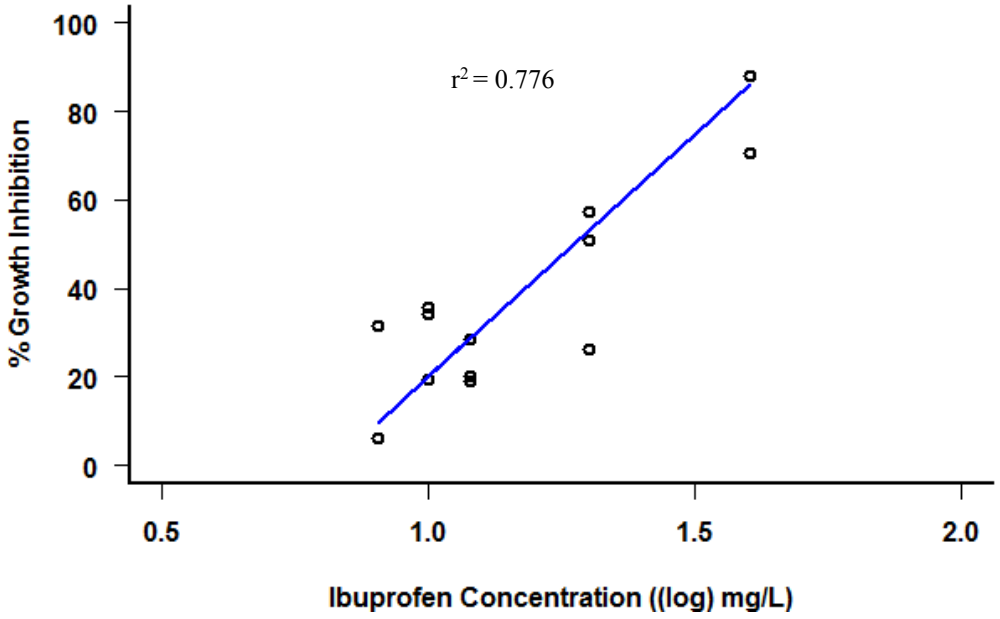

Figure 2.24 - Semi-log scatter plot of Ibuprofen concentration and percentage inhibition of growth for *Asterionella formosa* using regression analysis.

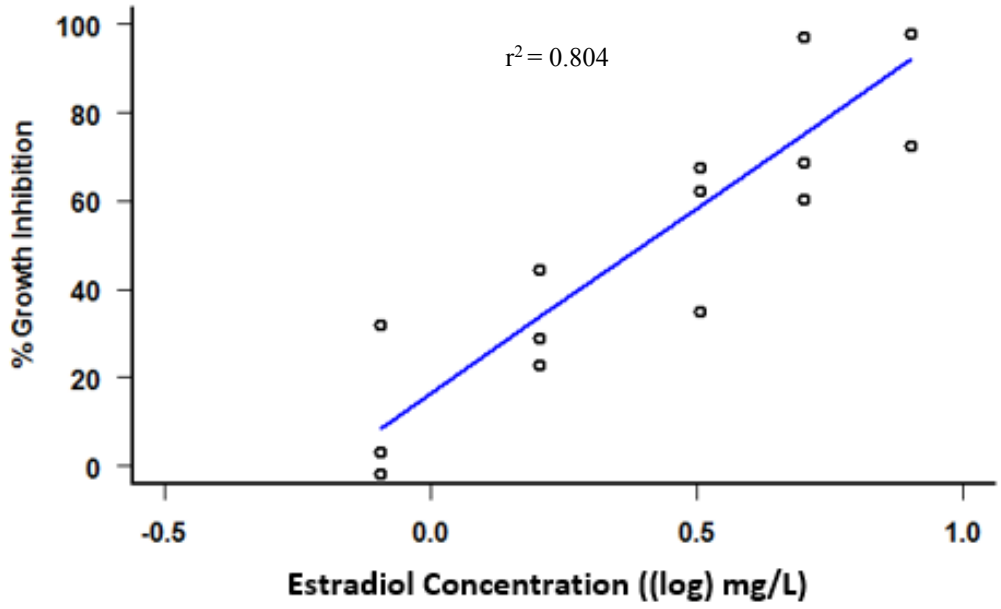

Figure 2.25 - Semi-log scatter plot of 17-b Estradiol concentration and percentage inhibition of growth for *Asterionella formosa* using regression analysis.

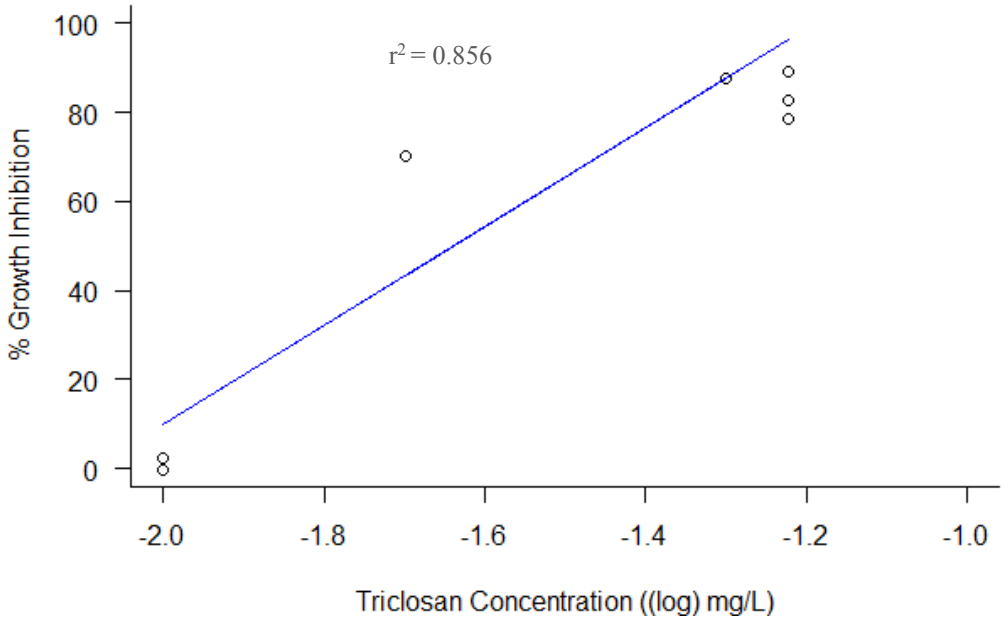

Figure 2.26 - Semi-log scatter plot of Triclosan concentration and percentage inhibition of growth for *Asterionella formosa* using regression analysis.

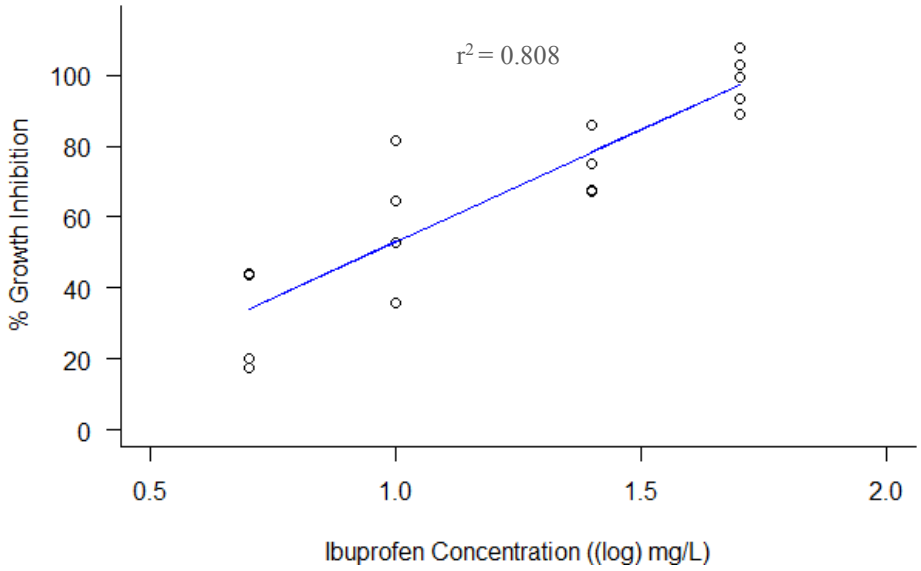

Figure 2.27 - Semi-log scatter plot of Ibuprofen concentration and percentage inhibition of growth for *Diatoma tenuis* using regression analysis.

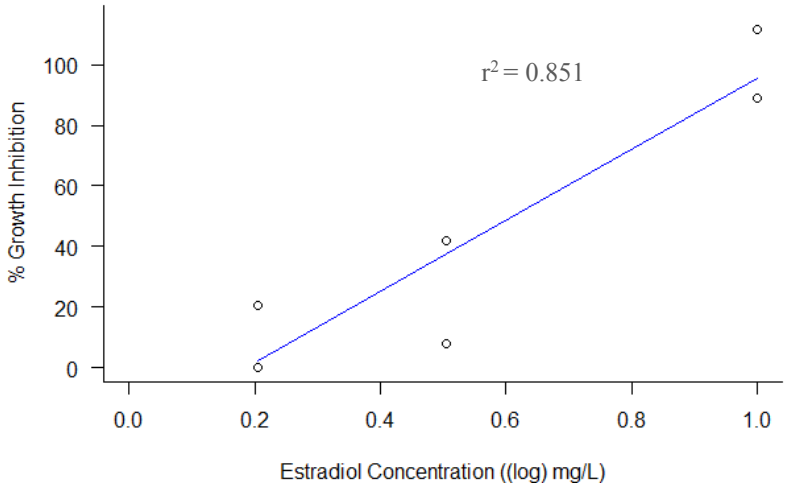

Figure 2.28 - Semi-log scatter plot of 17-β Estradiol concentration and percentage inhibition of growth for *Diatoma tenuis* using regression analysis.

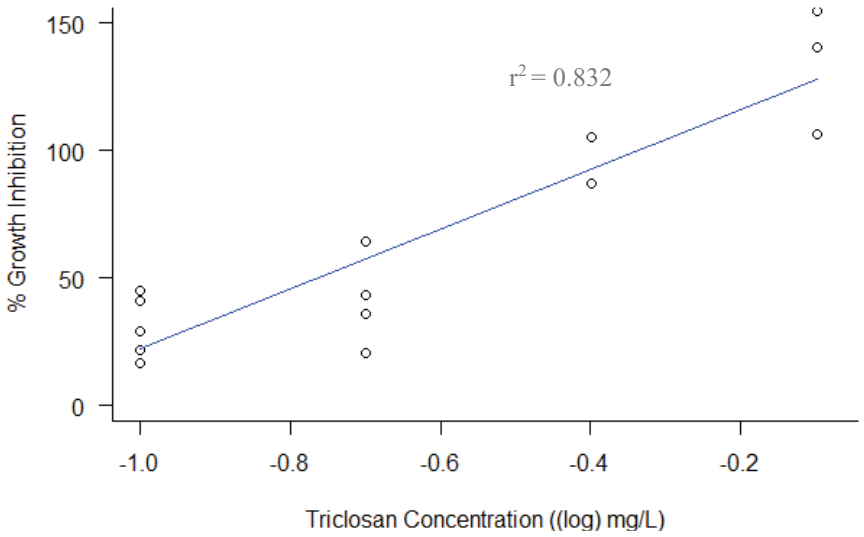

Figure 2.29 - Semi-log scatter plot of Triclosan concentration and percentage inhibition of growth for *Diatoma tenuis* using regression analysis.

Table 2.1 - Predicted toxicological values for individual testing.

PPCP Concentration (EC_x)	**Ibuprofen (mg/L)**		**17-β Estradiol (mg/L)**		**Triclosan (µg/L)**	
	A. formosa	***D. tenuis***	***A. formosa***	***D. tenuis***	***A. formosa***	***D. tenuis***
5	7.26	1.73	0.736	3.074	13	78
10	8.07	2.08	0.844	3.258	14	86
20	9.96	3.00	1.107	3.660	16	103
40	15.18	6.22	1.906	4.619	23	149
50	18.75	8.95	2.500	5.190	28	179
80	35.27	26.71	5.648	7.358	47	312

2.3.3 Mixture Toxicity Testing

Following the assessment of single toxicity of the three PPCPs, the effects of multiple toxicity exposure was studied. All three PPCPs were combined in a multicomponent mixture using the predictive values of their effective concentrations (EC_x). The multicomponent mixtures consisted of the following concentrations: EC_5, EC_{10}, EC_{20}, EC_{40}, and EC_{80}. The specific growth curves below (Figures 2.30 and 2.31) illustrate the effects of mixture toxicity on both diatoms.

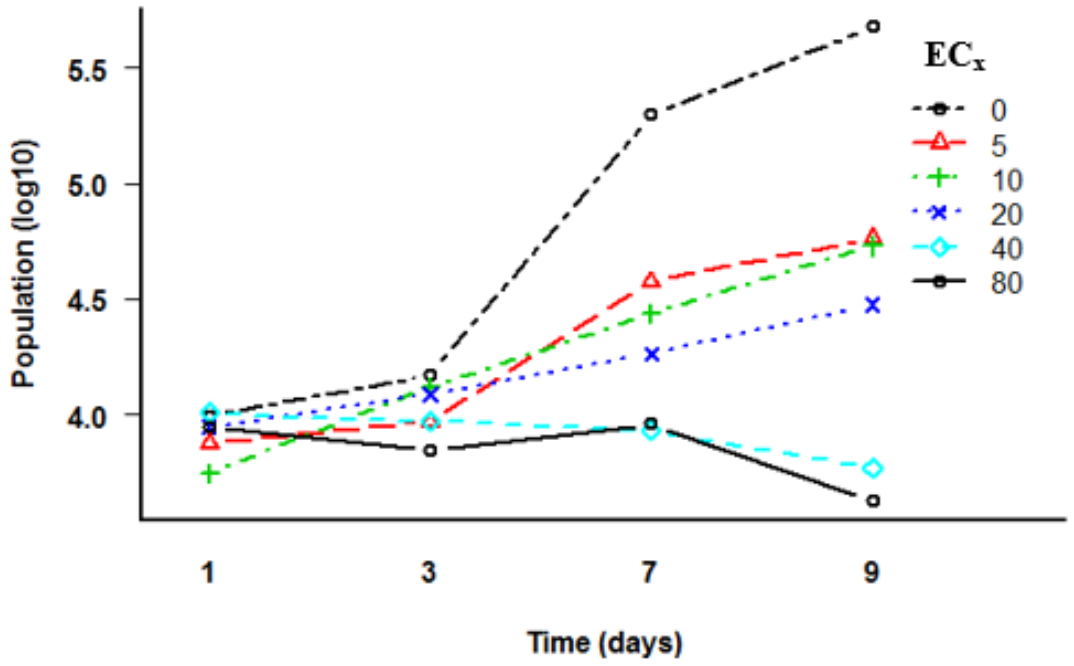

Figure 2.30 – An illustration of a Blocked ANOVA depicting growth responses of *Asterionella formosa* under exposure to the three PPCPs of study under a range of toxicological exposure.

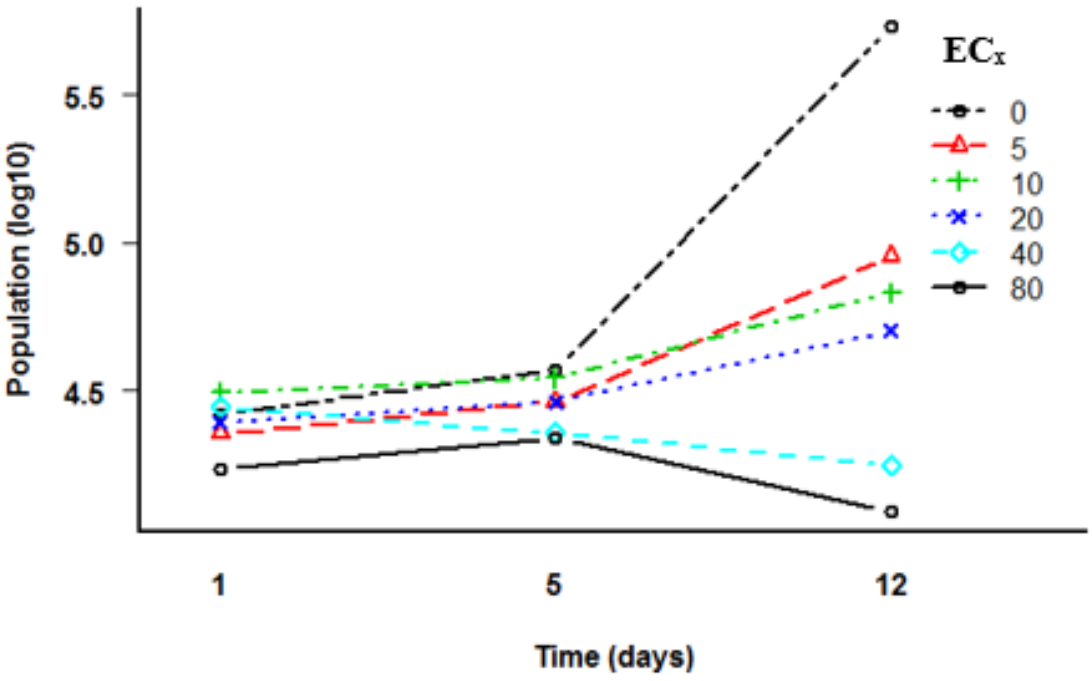

Figure 2.31 - An illustration of a Blocked ANOVA depicting growth responses of *Diatoma tenuis* under exposure to the three PPCPs of study under a range of toxicological exposure.

The exposure to all three PPCPs in combination proved to have significant effects on the growth of *A. formosa*, and *D. tenuis* ($F_{5,65}$ = 11.62, p = 4.85 x 10^{-8}, t = 9 and $F_{5,65}$ = 8.722, p = 2.27 x 10^{-6}, t = 12, respectively). The growth curves when exposed to the following concentrations (EC_x) showed significant differences (α = 0.05): *A. formosa* (0 - 5, 0 - 10, 0 - 20, 0 - 40, 0 - 80, 5 - 80, and 10 - 80) and *D. tenuis* (0 - 20, 0 - 40, 0 - 80, 5 - 80, and 10 - 80). The results from mixture toxicity is illustrated in the scatter plots below (Figures 2.32 and 2.33).

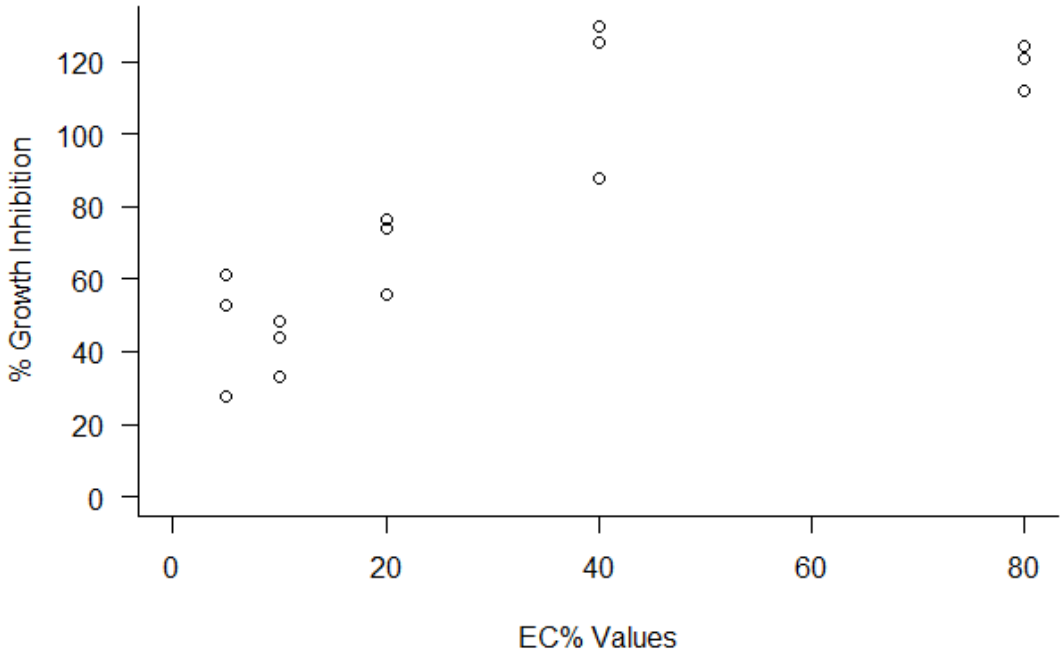

Figure 2.32 – A scatter plot illustration of percentage of growth inhibition with varying mixture toxicity concentrations (ECx) for *Asterionella formosa.*

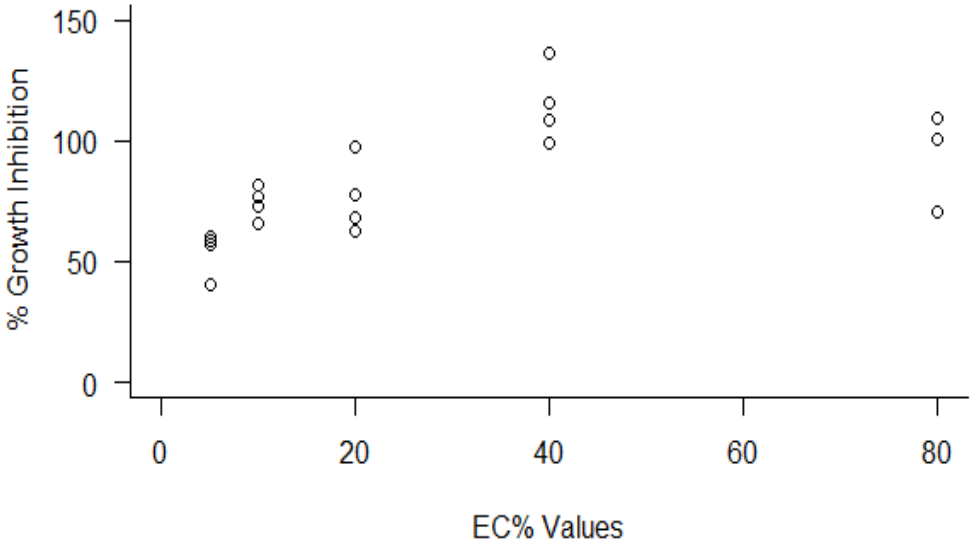

Figure 2.33 – A scatter plot illustration of percentage of growth inhibition with varying mixture toxicity concentrations (ECx) for *Diatoma tenuis*.

The concentration-response curves were transformed into semi-log plots to obtain a linear relationship between the two variables, $\log_{10} EC_X$ and percentage inhibition of growth (Figures 2.34 and 2.35). The slope of these linear correlations was then used to calculate predictive toxicological values (Table 2.2). These concentrations in all cases resulted in EC_x values higher than that of the predictive single toxicity values. For instance, 100% growth inhibition was evident at concentrations where individual exposure of each PPCP was only causing 40% growth inhibition. Exposure to all three compounds caused much greater growth inhibition for each diatom than exposure to only one PPCP at a time

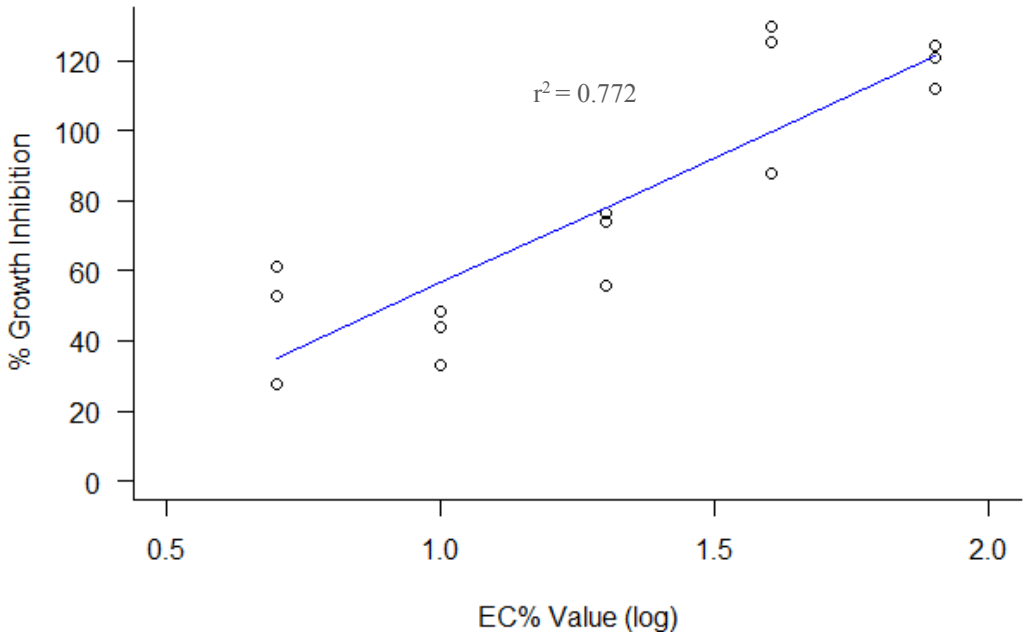

Figure 2.34 - Semi-log scatter plot of mixture toxicity concentrations (ECx) and percentage inhibition of growth for *Asterionella formosa* using regression analysis.

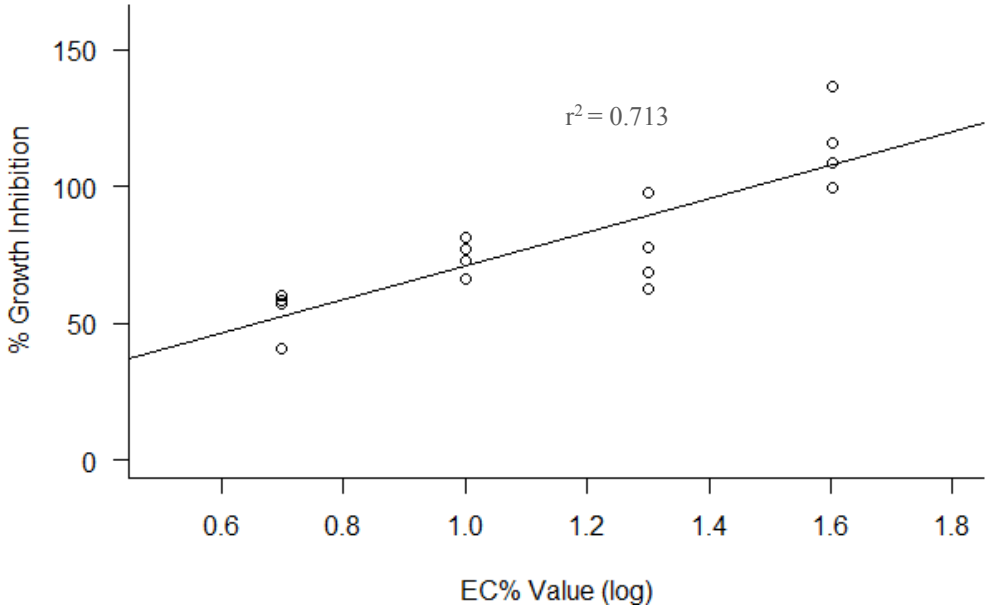

Figure 2.35 - Semi-log scatter plot of mixture toxicity concentrations (ECx) and percentage inhibition of growth for *Diatoma tenuis* using regression analysis.

Table 2.2 - Toxicological values causing x percentage of growth inhibition from mixture toxicity experiment using toxicological values from individual toxicity testing.

Concentration (EC_x) of Each Individual PPCP	Mixture Toxicity (EC_x)	
	A. formosa	***D. tenuis***
5	47.2 ± 17.5	54.1 ± 9.2
10	41.7 ± 7.9	74.3 ± 6.5
20	68.5 ± 11.3	76.6 ± 15.3
40	114.0 ± 22.9	115.1 ± 15.8
80	119.1 ± 6.29	110.8 ± 38.2

2.3.4 Optical Density

The changes in optical density with exposure to individual and mixture pharmaceuticals can be seen below (Figures 2.36 through 2.43). In all cases, optical density increased over the course of the experiment. A Blocked ANOVA revealed that exposure to Triclosan showed no significant effects on optical density in *A formosa* and *D. tenuis* ($F_{3,31} = 3.54$, $p = 0.057$ and $F_{4,34} = 2.015$, $p = 0.114$, respectively). Additionally, exposure to Ibuprofen, 17-β Estradiol, and mixture toxicity (COMBO) for both the diatom species resulted in a significantly lower level of optical density when compared to that of the controls ($\alpha = 0.05$). More specifically, significant effects were seen for *A. formosa*: Ibuprofen between 0 - 20 and 0 - 40 ($F_{3,31} = 6.197$, $p = 0.002$), 17-β Estradiol between 0 - 5, 0 - 8 and 0 - 12 ($F_{3,31} = 7.781$, $p = 0.001$), and COMBO between 0 - 5, 0 - 10, 0 - 20, 0 - 40, and 0 - 80 ($F_{5,65} = 11.62$, $p = 4.84 \times 10^{-8}$); and *D. tenuis*: Ibuprofen between 0 - 10 and 5 - 10 ($F_{2,20} = 5.759$, $p = 0.011$), 17-β Estradiol between 0 - 10 and 5 - 10 ($F_{2,14} = 7.729$, $p = 0.007$), and COMBO between 0 - 10, 0 - 20, 0 - 40, and 0 - 80 ($F_{5,65} = 8.722$, $p = 2.27 \times 10^{-6}$).

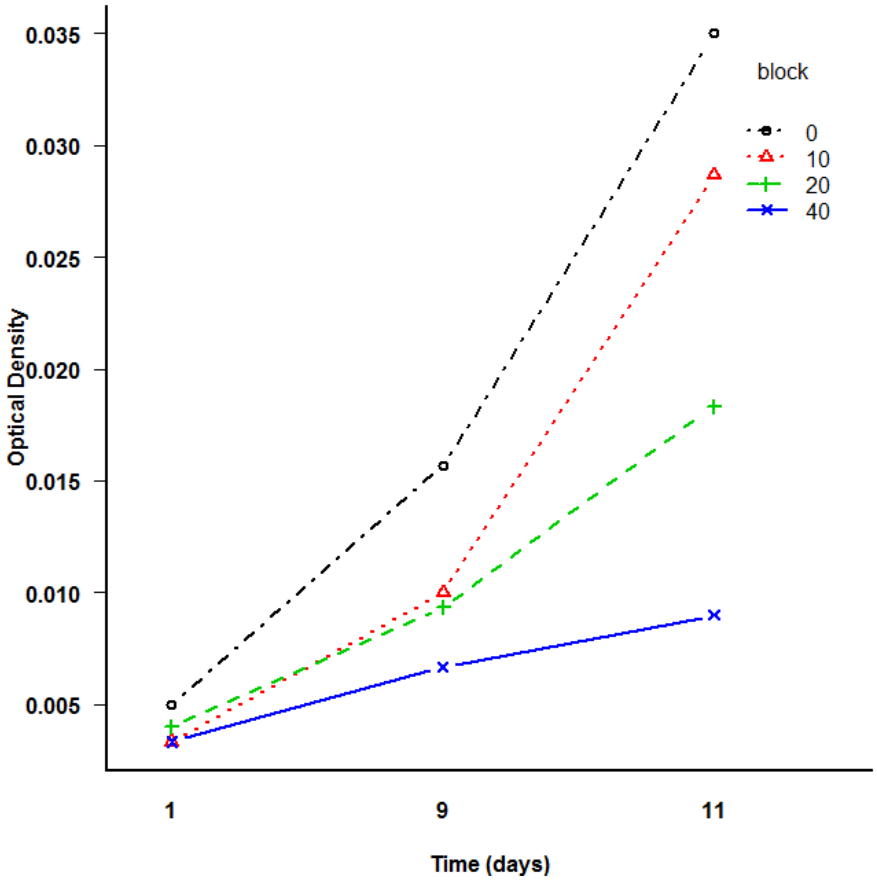

Figure 2.36 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Asterionella formosa* with Ibuprofen.

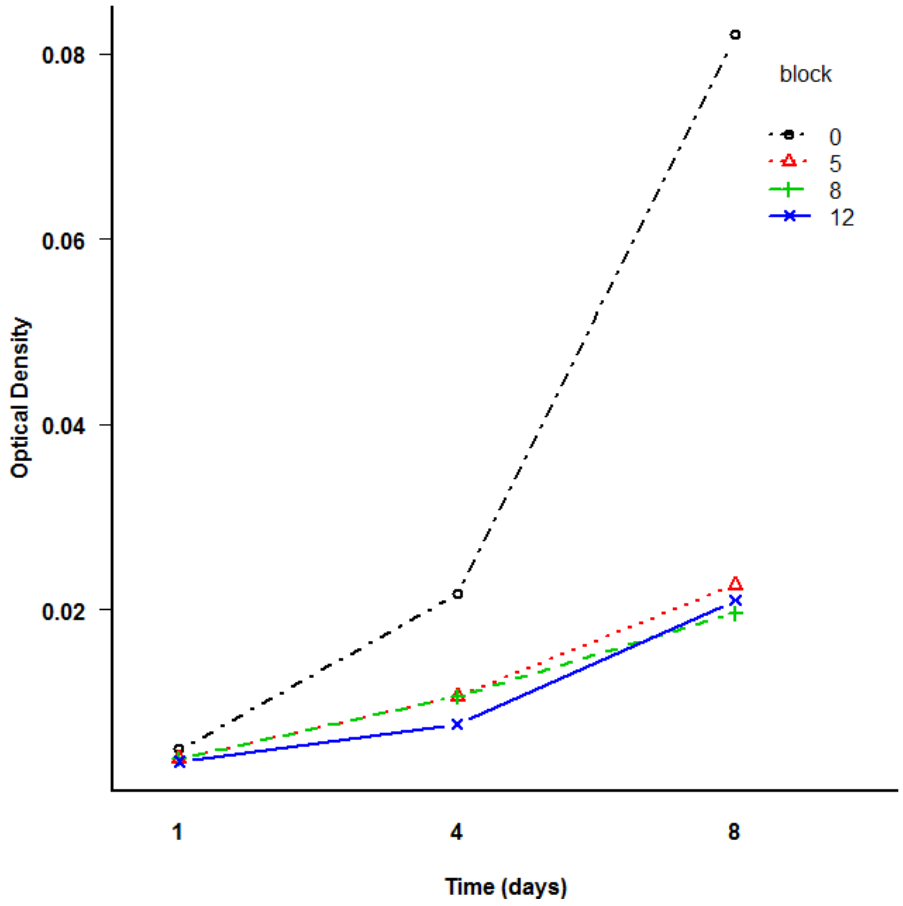

Figure 2.37 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Asterionella formosa* with 17-β Estradiol.

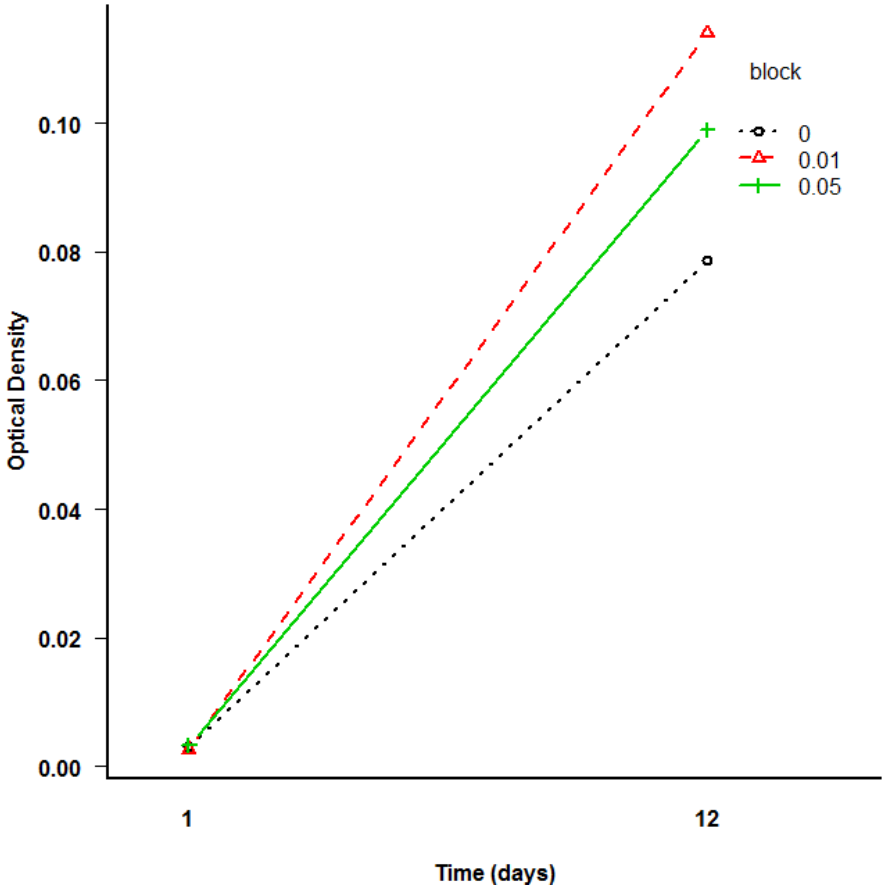

Figure 2.38 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Asterionella formosa* with Triclosan.

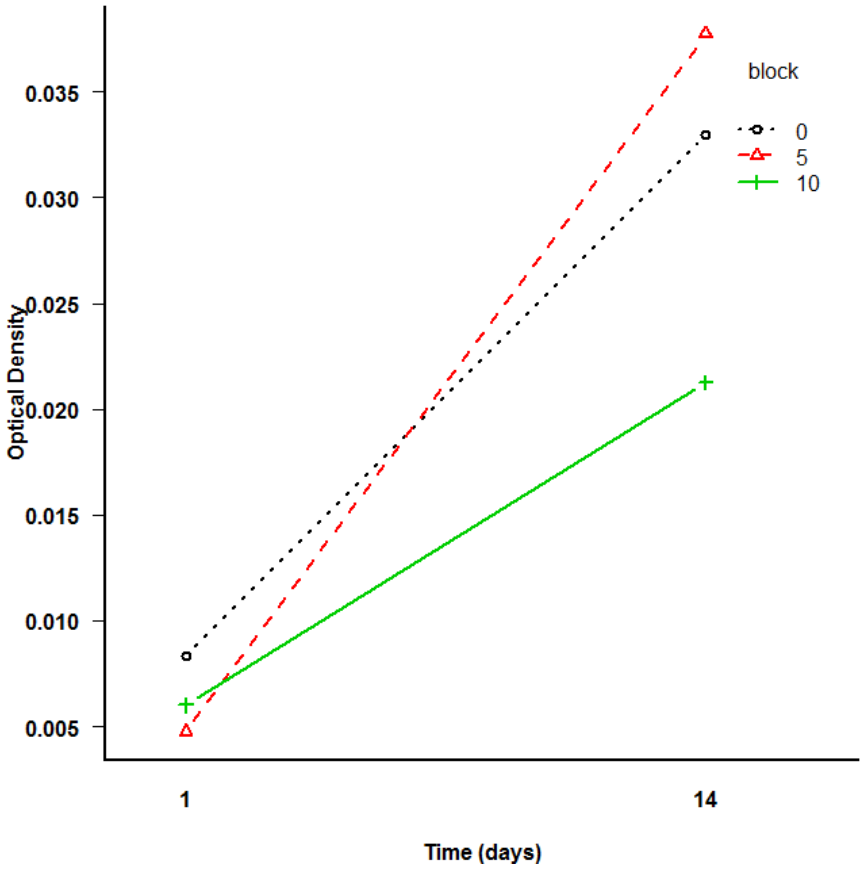

Figure 2.39 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Diatoma tenuis* with Ibuprofen.

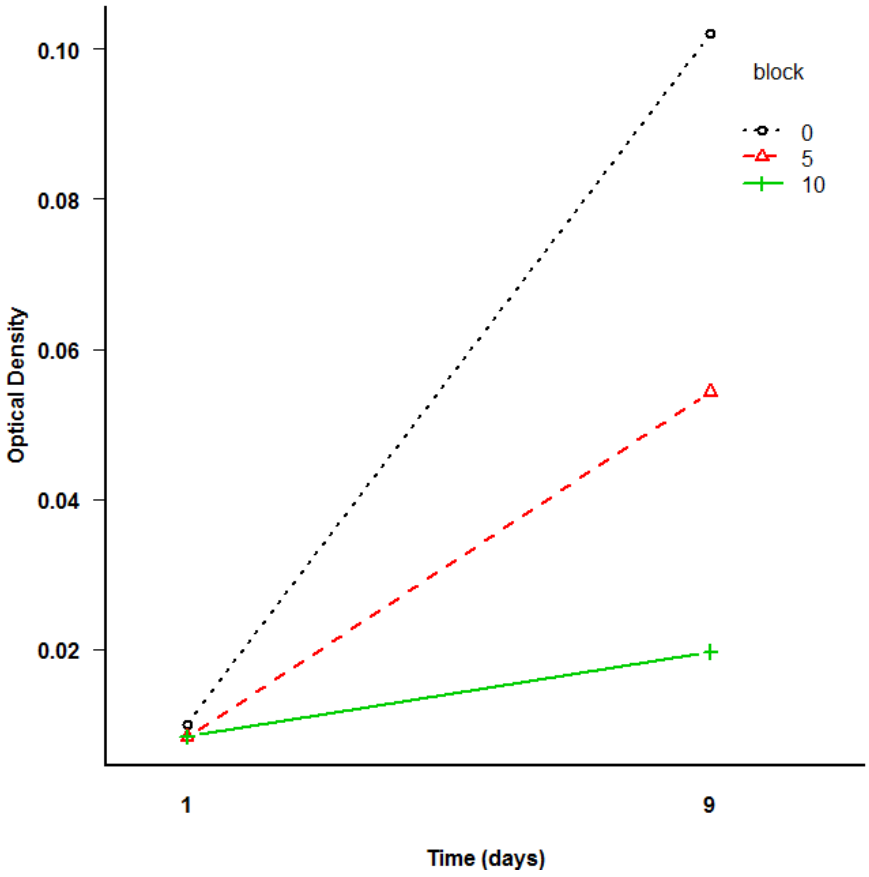

Figure 2.40 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Diatoma tenuis* with 17-β Estradiol.

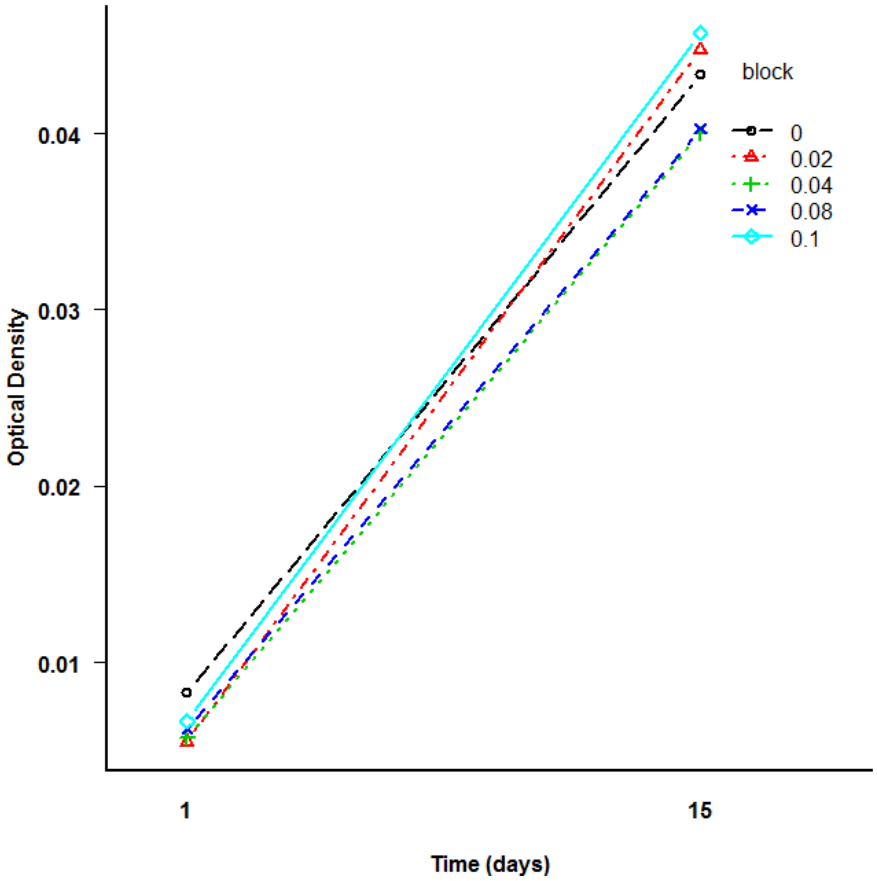

Figure 2.41 - An illustration of a Blocked ANOVA comparing changes in optical density during individual toxicity testing of *Diatoma tenuis* with Triclosan.

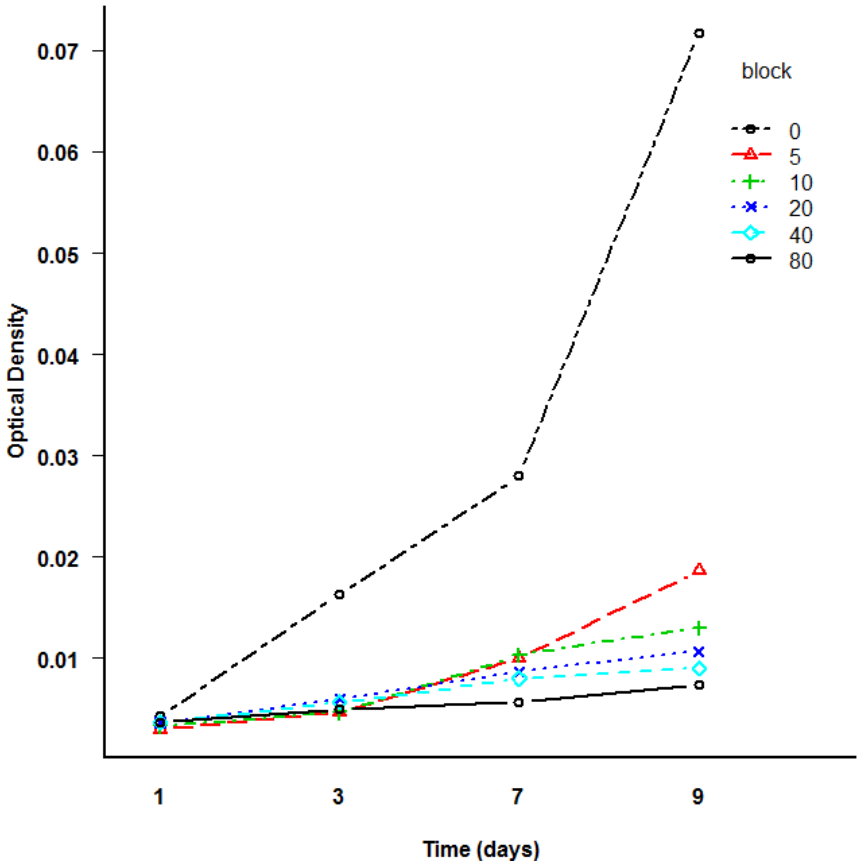

Figure 2.42 - An illustration of a Blocked ANOVA comparing changes in optical density during mixture toxicity testing with *Asterionella formosa.*

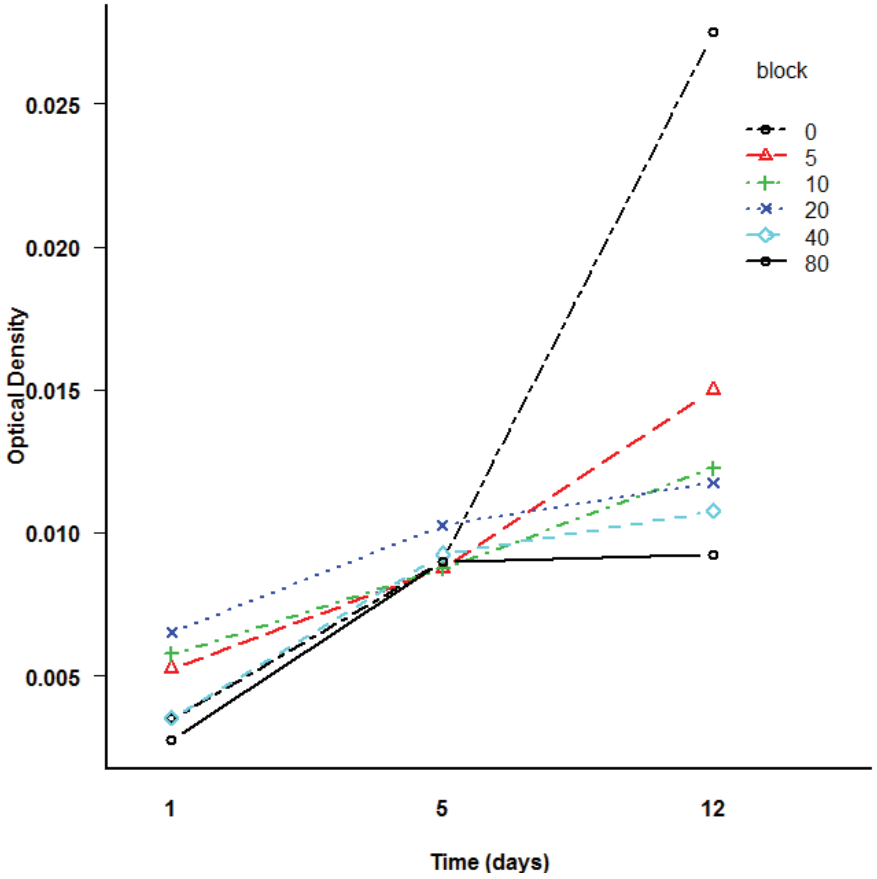

Figure 2.43 – An illustration of a Blocked ANOVA comparing changes in optical density during mixture toxicity testing with *Diatoma tenuis*.

2.4 Discussion

2.4.1 Single Toxicity Testing

This study revealed an EC_{50} value of Ibuprofen for *A. formosa* and *D. tenuis* to be 18.7 and 8.95 mg/L (Table 2.1). These concentrations are much lower than that of the EC_{50} value for micro green algae (41.13 mg/L), according to ECOSAR database (US EPA, 2016). Unfortunately, very little data currently exists pertaining to the toxicological concentrations of Ibuprofen on micro-algae. Current articles, however, do suggest that this drug has a strong potential of stimulating the growth rate of microalgae at low concentrations (<1000 µg/L), and that the mechanism of action was likely nonpolar narcosis, relating to hydrophobicity (Cleuvers, 2004; Pomati et al., 2004).

The EC_{50} value of 17-β Estradiol for *A. formosa* and *D. tenuis* is 2.5 and 5.2 mg/L, respectively. The ECOSAR database provides a value quite similar to these for micro green algae (EC_{50} = 4.278 mg/L) (US EPA, 2016). Other studies also reported similar toxicological values for this pharmaceutical on several microalgal species. For instance, two micro green algal species, *Pseudokirchneriella subcapitata* and *Desmodesum subspicatus*, are believed to have an EC_{50} value of 0.87 and 1.07 mg/L for 17- β Estradiol, respectively (Salomão et al., 2014). The diatom, *Navicula incerta*, was found to have EC_{50} value of 10 mg/L (Y. Liu et al., 2010). Moreover, recent literature has found that similar to Ibuprofen, 17-β Estradiol has the ability to enhance cell growth of micro-algae at low concentrations (Hom-Diaz et al., 2015; F. Wu et al., 2014). As previously mentioned, this PPCP has a moderately high octanol-water partitioning coefficient. The fate of Estradiol was examined after exposing two species of green algae, *Pseudokirchneriella sp.* and *Chlamydomonas sp.*, to > 5 mg/L of this compound. This resulted in more than 50% removal of the PPCP due to adsorption onto the algal biomass (Hom-Diaz et al., 2015). This has strong implications as to the bioaccumulation impacts of 17-β Estradiol in our inland waters.

The two diatom species in this study proved to be the most sensitive to Triclosan. The results indicate that EC_{50} values for *A. formosa* and *D. tenuis* are 32 and 18 µg/L, respectively. According to the ECOSAR database, the EC_{50} value for micro green algae is much higher (1.443 mg/L) (US EPA, 2016). A study on the impact of Triclosan on micro green alga, *Scendesmus subspicatus*, and the diatom, *Skeletonema costatum*, showed an EC_{50} value of 0.7 and 66 µg/L, respectively (Franz et al., 2008). A different species of micro green alga, *Scendesmus sp*., was found to have an EC_{50} value of 1.4 µg/L of Triclosan (Orvos et al., 2002). Triclosan has been shown to impact multiple target points of algal cells using narcosis, uncoupling mode-of-action, among other mechanisms (Franz et al., 2008; Lawrence et al., 2015; Wang et al., 2013). The results include, but are not limited to, disruption of membrane metabolism and destruction of chloroplasts (Johansson et al., 2014; Lawrence et al., 2015; Wang et al., 2013). In addition, the antimicrobial properties of Triclosan have been shown to impact greatly on benthic communities, specifically, the biomass of cyanobacteria (Drury et al., 2013; Johansson et al., 2014; Lawrence et al., 2015).

2.4.2 Mixture Toxicity Testing

The individual toxicity tests provided some idea about the toxicological values of mixture toxicity testing of these pharmaceutical compounds. The PPCPs, amongst many others, are commonly found in the wastewater effluents in North America and as such, it was thought necessary to explore the synergistic effects of these compounds on microalgae.

Multiple toxicity testing showed more extreme impacts on the growth of *A. formosa* and *D. tenuis*, when compared to the impacts of individual toxicity testing. The EC_{100} values for each diatom during mixture toxicity testing was well below their respective EC_{50} values from the individual toxicity testing of each PPCP (Table 2.2). This vast difference in toxicological effect between single and mixture exposure is important to note, as current risk assessment values and hazard quotients for the freshwater systems of North America are typically based on these contaminants being tested individually, rather than in combination.

The effects of mixture toxicity of PPCPs in our inland waters has only recently been explored (Cleuvers, 2004; Geiger, 2014; Ginebreda et al., 2014). Two species of green algae, *Desmodesmus sp.* and *Chlorella vulgaris*, were found to have an increased sensitivity to mixture toxicity of nonsteroidal anti-inflammatories (NSAIDs) than individual exposure of those compounds in separate studies (Cleuvers, 2004; Geiger, 2014). The toxicological range was 72 to 626 mg/L for *Desmodesmus sp.* and the impacts seen in both studies were sought to be the result of the concept of concentration addition (Cleuvers, 2004; Geiger, 2014). It is commonly seen that the combined effect of a chemical mixture is going to be higher than that of an individual effect when it comes to micro-algae (Cizmas et al., 2015; Cleuvers, 2004; Geiger, 2014; Ginebreda et al., 2014).

2.4.3 Optical Density

Optical density followed the same trend for *A. formosa* and *D. tenuis*. Exposure to Ibuprofen and 17-β Estradiol, resulted in negative impacts to optical density, whereas, Triclosan showed no significant effect on the optical density of these species within test concentrations (0 to 3.2 µg/L). Optical density is an excellent surrogate measurement for chlorophyll production in microalgae (Yentsch et al., 1963). Many previous works have shown decreases in chlorophyll production of algae when exposed to toxic concentrations of particular chemicals (Bácsi et al., 2016; Cleuvers, 2004; Eriksson et al., 2014; Geiger, 2014; Hunt, 2006; B. Liu et al., 2011; Wang

et al., 2013). It is thought that this disruption of chlorophyll production is caused by morphological and ultra-structural alterations consistent with the induction of a stressful condition (Moro et al., 2014). Triclosan, however, exhibited no significant impacts on the optical density of either species of this study. Other works are not in accordance with these findings, where they have studied the effects of Triclosan on species of green algae (Eriksson et al., 2014; Hunt, 2006; Wang et al., 2013). This suggests that diatoms could potentially be less sensitive to the membrane disruption that correlates with the exposure of Triclosan as reported for micro green algae (Eriksson et al., 2014; Hunt, 2006; Wang et al., 2013).

2.5 Conclusion

Microalgae have been studied extensively as bio-indicators for aquatic health (Julius et al., 2007; Moro et al., 2014). Recent studies indicate that diatoms could be useful indicators of PPCP contamination (Julius et al., 2007; Moro et al., 2014). In this study, the environmental limitations of 17-β Estradiol, Ibuprofen, and Triclosan on two diatoms, *A. formosa* and *D. tenuis*, were explored. Concentration-dependent responses of PPCPs were found for both diatoms ranging from 0 to 100 % growth inhibition. The two diatoms in this study were more sensitive to the toxicological effects of Ibuprofen and Triclosan than that of previously studied green micro-algae (US EPA, 2016). *A. formosa* had a reported EC_{50} for 17-β Estradiol lower than recent studies on micro-green algae and other diatoms, whereas, the toxicological effects on *D. tenuis* was more predictable based on previous literature (Y. Liu et al., 2010; US EPA, 2016). The combined effect of Ibuprofen, 17-β Estradiol, and Triclosan was greater than exposure to individual compounds for *A. formosa* and *D. tenuis*. Optical density of the culture was significantly inhibited when exposed to Ibuprofen and 17-β Estradiol within test concentrations for the two diatom species, however, Triclosan caused no effect. A review of this study indicates that *A. formosa* and *D. tenuis* are more sensitive to the three PPCPs than some micro-algae from other communities and as such, suggests that diatoms could function well as an indicator for pharmaceutical contamination in Lake Simcoe. In addition, mixture toxicity resulted in compounding effects, in accordance with previous studies (Cleuvers, 2004; Geiger, 2014; Ginebreda et al., 2014).

3. Investigating the Suitability of Diatoms as an Indicator of PPCP Contamination in Lake Simcoe: A Case Study

3.1 Introduction

Pharmaceuticals and personal care products (PPCPs) are a unique group of emerging environmental contaminants entering our freshwater systems. Many of these compounds are known to pose risk to aquatic organisms at all trophic levels (Bácsi et al., 2016; Nesbitt, 2011; Parolini, 2010). However, data pertaining to the effects of PPCPs on the primary producers is still significantly lacking, Recent studies have identified an influx of PPCPs in Lake Simcoe (Metcalfe, 2014; Ontario, 2014; Wray et al., 2014). This study aims to explore the occurrence of three PPCPs in the waters of Lake Simcoe surrounding three Waste Water Treatment Plants (WWTPs) in comparison with a control site located further away from any of the WWTPs. Estrone, Ibuprofen, and Triclosan have been selected for study based on their prevalence as reported in previous studies on Lake Simcoe and Great Lakes. In addition, these contaminants are all considered to be lipophilic ($\log K_{ow} > 4$), which can influence accumulation in biota. The WWTPs in this study, discharge their effluent into creeks that feeds into the lake. The hydrologic conditions, PPCP concentrations, and microalgal composition were assessed at the point of discharge and the point of confluence with the lake. My hypothesis is that microalgae community composition can be used as an indicator of the levels of residual PPCPs in wastewater effluent. Biological monitoring of PPCP contamination using microalgae would provide a fast and inexpensive alternative to conventional laboratory measurements. Diatoms are an abundant and diverse community of microalgae that have long been studied for their usefulness as indicators of environmental conditions (i.e. nutrient-loading, trace metals, organic contamination, etc.) (Smol & Stoermer, 2010). Therefore, the results of this study may suggest the usefulness of the diatoms species to act as an indicator of PPCP contamination in Lake Simcoe.

3.2 Methodology

3.2.1 Sampling Sites

Water samples were collected from two points outside of three WWTP's that discharged treated effluents into a creek or a river that fed into Lake Simcoe (Figure 3.1). In addition, I also sampled two points outside of a control location that was hydrologically similar to the WWTP

sites but did not receive any effluent. The first sampling point (upstream point) at each site was the point at which the effluent first entered the creek or river water. The second sampling point (downstream point) was located at the mouth of the river or creek where it joined Lake Simcoe. Sampling was conducted on August 15, 2017 between 0900 and 1700. The temperature during the sampling period ranged from 18 to 26 ºC, with a cloud cover of 0 - 40%, and zero precipitation.

Figure 3.1 - A map of Lake Simcoe and surrounding municipalities, with each sampling point indicated by the yellow markers.

3.2.1.1 Bradford West Gwillimburry

Bradford West Gwillimburry (BWG) consists of approximately 35,000 people and its WWTP is situated along the shores of the West Holland River. The WWTP also uses traditional

tertiary treatment of UV disinfection and a deep bed sand filtration system to aid in the purification of the effluent.

The upstream point (Figure 3.2) had rocky substrate with a shallow, narrow (~ 2 m) bank width and 15 cm water depth. The composition of this creek was approximately 90 - 98% treated effluent at any given time, with the remaining contributed by the runoff. The creek ran for approximately 700 m before converging with the West Holland River. This river flowed for nearly 11 km before reaching the most southern bay of Lake Simcoe. Much of this path fell within the Greenbelt Natural Heritage System and more specifically, transversed the Holland Marsh. The point at which the West Holland River meets Lake Simcoe was the downstream point (Figure 3.3). This river opening was broad, approximately 150 m wide and 7.5 m deep.

Figure 3.2 – The upstream site at the BWG sampling location.

Figure 3.3 – The downstream site at the BWG sampling location.

3.2.1.2 Orillia

The City of Orillia has approximately 30,000 people and is located between Lake Couchiching and Lake Simcoe. The WWTP of this city is a conventional activated sludge plant with primary settling, UV disinfection, and anaerobic digestion systems. Effluent discharge flows into a small creek that leads to Lake Simcoe. Notably, this location includes a hospital within the city limits that would contribute to the discharge, and the treatment plant is directly adjacent to a landfill. As such, there is potential for leachate to enter the waters and sediments along with the effluent.

This WWTP was located in a wetland. The upstream sampling point (Figure 3.4) was from an area with an approximately 2 m bank width and 45 cm depth. The creek meandered through the wetland for 0.5 km before entering Lake Simcoe. The downstream sampling point (Figure 3.5) was a reach with about 70 m bank width and 0.5 m depth. This location was frequently inhabited by several (50+) Black Cormorants, which dropped their feces into the creek leaving a film covering much of the water surface.

Figure 3.4 – The upstream site at the Orillia sampling location.

Figure 3.5 – The downstream site at the Orillia sampling location.

3.2.1.3 Lagoon City

Lagoon City is a small community situated on a 15 km system of man-made water canals, in the Township of Ramara. The WWTP of Lagoon City is a traditional plant, similar to that of the Orillia WWTP, however, it only serves around 1200 people and is thus much smaller in size. The upstream sampling point (Figure 3.6) had an approximately 1.5 m bank width and 35 cm depth. The creek then flowed through a wetland for more than 2.5 km before converging with Lake Simcoe. The downstream sampling point (Figure 3.7) had an approximately 30 m bank

width and 1 m depth. Unlike the other points of convergence with the lake, this location had a slow flow.

Figure 3.6 – The upstream point at the Lagoon City sampling location.

Figure 3.7 – The downstream site at the Lagoon City sampling location.

3.2.1.4 Brechin

The two control sites were located in the Town of Brechin, on the Talbot River. These sites were chosen because they appeared to be hydrologically similar to the other sampling locations but were far from any municipal WWTP. The upstream sampling point (Figure 3.8) had an approximately 1.5 m bank width and 30 cm depth. The creek traversed a wetland for 2 km

before converging with Lake Simcoe. The downstream sampling point (Figure 3.9) had an approximately 20 m bank width and 5.5 m depth. This location had a slower movement of water in comparison to the BWG and Orillia sampling locations. However, both sampling points of the control site exhibited similar physical and chemical parameters (i.e., dissolved oxygen, temperature, conductivity, etc.), and vegetative characteristics as the other three locations. The creeks at all locations had a protected (Provincially Significant) wetland between their upstream and downstream sampling sites.

Figure 3.8 – The upstream point at the control site sampling location.

Figure 3.9 – The downstream point at the control site sampling location.

3.2.2 Environmental Parameters

Field sampling consisted of measuring pH, conductivity, dissolved oxygen, temperature, total suspended solids (TSS), total phosphorus (TP), nitrate, and PPCP concentrations (Ibuprofen, Estrone, and Triclosan).

A multiparameter hydrolab (VWR SympHony, SB90M5) was used to measure pH and conductivity. A separate hydrolab (Hach, HQ40D), specific to measuring oxygen in the water was used for dissolved oxygen, and temperature measurements.

Four 1 L and one 125 mL polyethylene bottles were used to collect water samples 10 cm below the surface, centered between the banks of each sampling point. The 1 L bottles were used for TSS (1), PPCP concentrations (2), and algal samples (1), whereas the 125 mL bottle was used for nutrient (TP and nitrate) analysis. All bottles were opaque and kept in a cooler box to reduce photo- and thermal degradation of samples.

3.2.2.1 TSS

A 1 L water sample was filtered through a Büchner funnel by vacuum pressure, passing through a 1 μm porous glass-fiber filter paper (Sigma-Aldrich). The filter paper was then carefully transferred into a drying chamber to dry at 40º C for 24 - 48 hours. The difference in weight of the filter paper before and after filtering and drying (± 0.0001 g) was taken as the TSS measurement.

3.2.2.2 PPCP Concentrations

The three PPCPs were analyzed from water collected in 1 L polyethylene bottles. The samples were collected in duplicate, and were immediately sent to a laboratory for analysis (Metcalfe laboratory, Trent University, Peterborough, Ontario). The compounds were extracted from the water samples and analyzed using liquid chromatography and tandem mass spectrometry. The details of these procedures were provided by Metcalfe:

Solid Phase Extraction

Water samples were extracted using Oasis MAX anion exchange cartridges, essentially as described by Li et al. (2010). The cartridges were pre-conditioned with methanol, followed by 0.1 M NaOH and then water (pH 8.0). Water samples (200 ml) were adjusted to pH 8 with 1.0% ammonium hydroxide and then loaded onto the cartridges after addition (100 µl) of an internal

standard mixture consisting of stable isotope surrogates for each target analyte (1 µg/ml of each compound). After loading of the water samples onto the Solid Phase Extraction (SPE) cartridges, they were eluted sequentially with 2 ml methanol and then 3 x 3 ml of 2% formic acid in methanol. The extracts were evaporated to near dryness and then reconstituted in 0.4 ml methanol. The recoveries of the target analytes using this SPE procedure were all > 75%.

Analysis

The analytes were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) in negative ion mode using an Applied BioSystems Sciex Q-Trap 5500 instrument with an electrospray ionization source, equipped with an Agilent 1100 series (ABS Sciex, Mississauga, ON, Canada) separation system and auto sampler. The analytes were separated chromatographically using a Genesis C-18 column and a guard column of the same stationary phase (Chromatographic Specialties, Brockville, ON, Canada). Multiple reaction mode (MRM) detection was performed using the precursor and product ion transitions for the target analytes and their stable isotope labelled surrogates. An external standard method with a five-point calibration curve was used for quantification, and the data were adjusted according to response of the surrogate internal standards. The Limits of Detection (LODs) and Limits of Quantitation (LOQs) were determined as the analyte concentration that produced a peak with a signal-to-noise ratio of 3:1 and 10:1, respectively, determined by analysis of serial dilutions of the analytical standard. Procedural blanks with deionized water were prepared for every five field samples.

3.2.2.3 Nutrients

The 125 mL frozen samples were thawed in a water bath and analyzed for both the nutrients on the same day. In preparation for the analysis, all glassware was washed with a 10% solution of hydrochloric acid. In addition to the field samples being measured, an extra sample was prepared with 100% de-ionized water to act as a control. The concentrations of total phosphorus and nitrate were measured using standard methods as outlined by Eaton et al. (2005).

Selected samples were shipped to Lakehead University Environmental Laboratory for phosphorus and nitrate analysis and to compare (verify) with the results obtained by using HACH pillows. The results showed very similar readings for the samples.

3.2.3 Microalgae Analysis

One-liter water samples from 10 cm below the surface were collected from each sampling point to analyze microalgae. The samples were preserved at 4ºC to minimize cell death and activity. The samples were concentrated by repeated centrifugation (2500 RPM for 15 mins) and cells were enumerated and identified using a counting chamber (hemocytometer) under a compound microscope at 40x and/or 100x magnification. The microalgae cells were identified to the genus in all cases, and species on all possible occasions. From these data, density, species richness, and species diversity were calculated.

Density of each species was calculated from the cell counts and expressed as # of cells/L. Species richness consisted of the total number of species from each sampling point. Species diversity was measured using the Shannon-Wiener Diversity Index (SI) (Equation 1); where *H* is the species diversity of the community, *S* is the species richness, and p_i is the proportion of *S* made up of the *i*th species. The proportion of cells which belong to the class Bacillariophyceae was calculated by tallying the number of species from said group and dividing it by the total cell count from that sample.

$$H = - \sum_{i=1}^{S} p_i \circ \ln(p_i) \quad (1)$$

3.2.4 Statistical Analyses

3.2.4.1 Regression Analysis

All algae, nutrient, physico-chemical, and PPCP data were tested for normality using Kolomogorov tests (R Core Team, 2013), and transformed using log(10), ln, or exponential functions as required. Parametric linear regression was used to determine the relationships between algae characteristics and predictor variables such as the physico-chemical, nutrient, and PPCP data.

3.2.4.2 Ordination Analysis

A gradient analysis method, redundancy analysis (RDA) using CANOCO software ver. 5, was used to distinguish the algal and environmental parameters and to develop a predictive model. This model was used to predict a linear combination of the environmental parameters from a linear combination of the algal parameters. Various biological parameters such as species richness, species diversity, species density, and % diatom, and presence of individual species

was assessed for their ability to act as indicators of water quality variables and/or PPCP concentrations.

3.3 Results

3.3.1 Environmental Parameters

Water temperature and pH were similar amongst all locations and sites, ranging from 20-24º C, and 7.0-7.8 pH units, respectively (Figure 3.10). Dissolved oxygen measurements were lower at the downstream sampling points compared to upstream for all locations except for BWG (Figure 3.11). The greatest difference between an upstream and a downstream site was at the Lagoon City location. Conductivity measurements were higher upstream compared to downstream at all locations, with the highest overall values seen at the BWG location and the lowest values at the control location (Figure 3.12). Total suspended solids were higher at the downstream compared to the upstream sites at all locations (Figure 3.13). The control site had relatively high amounts of TSS when compared to the other locations. Concentrations of nitrate at the upstream point were more than twice the downstream point at all locations (Figure 3.14). Total phosphorus (TP) concentrations exceeded the provincial guideline (20 µg/L). The TP values were quite similar amongst the WWTP locations ranging between 73 and 96 µg/L, whereas these concentrations were well above 200 µg/L at both of the control sampling points.

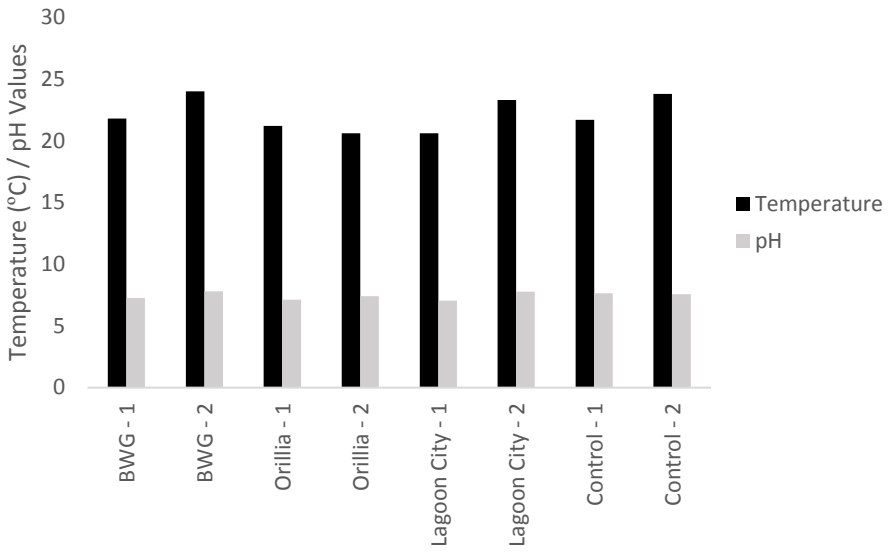

Figure 3.10 - Temperature and pH measurements at each sampling point, where upstream and downstream sites are denoted by '1' and '2'.

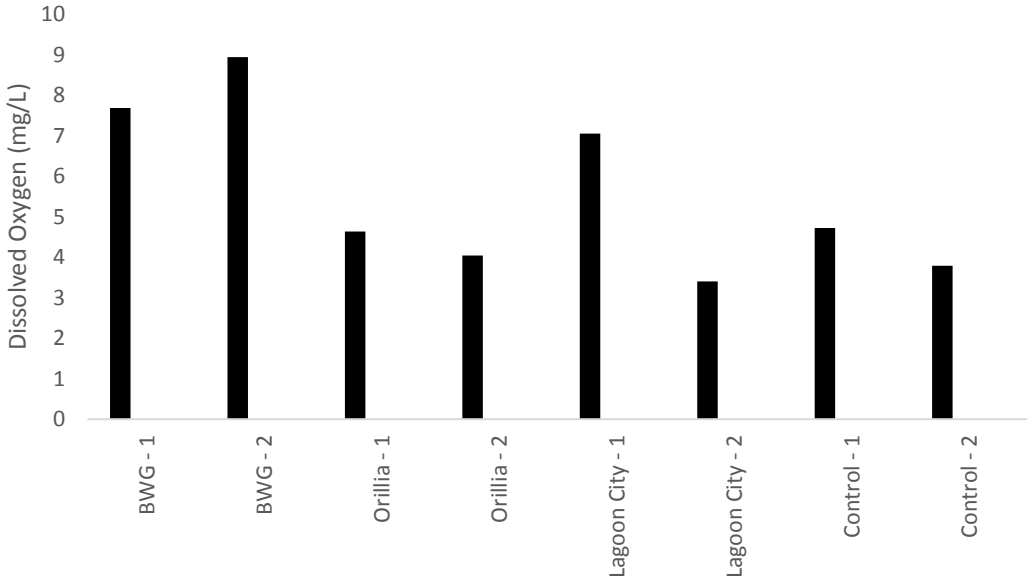

Figure 3.11 - Dissolved oxygen measurements at each sampling site, where upstream and downstream sites are denoted by '1' and '2'.

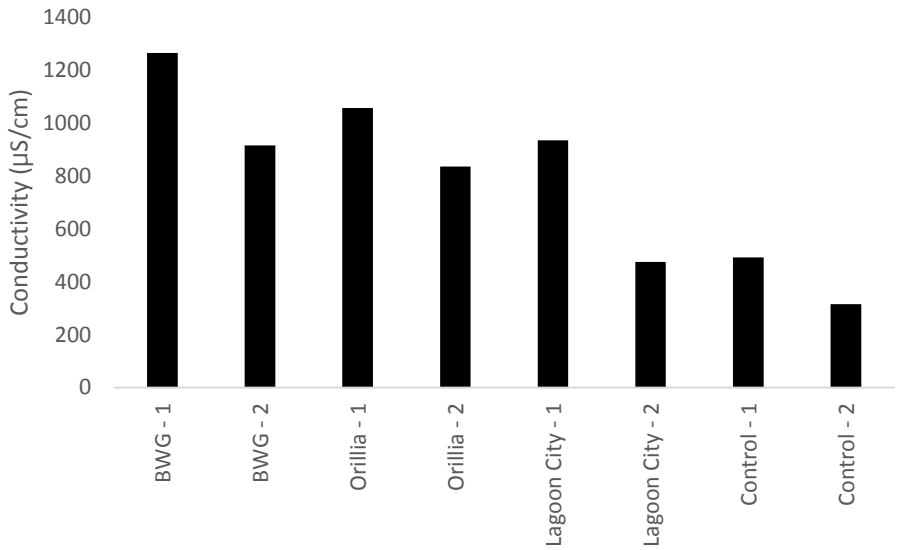

Figure 3.12 - Conductivity measurements at each sampling site, where upstream and downstream sites are denoted by ‘1’ and ‘2’.

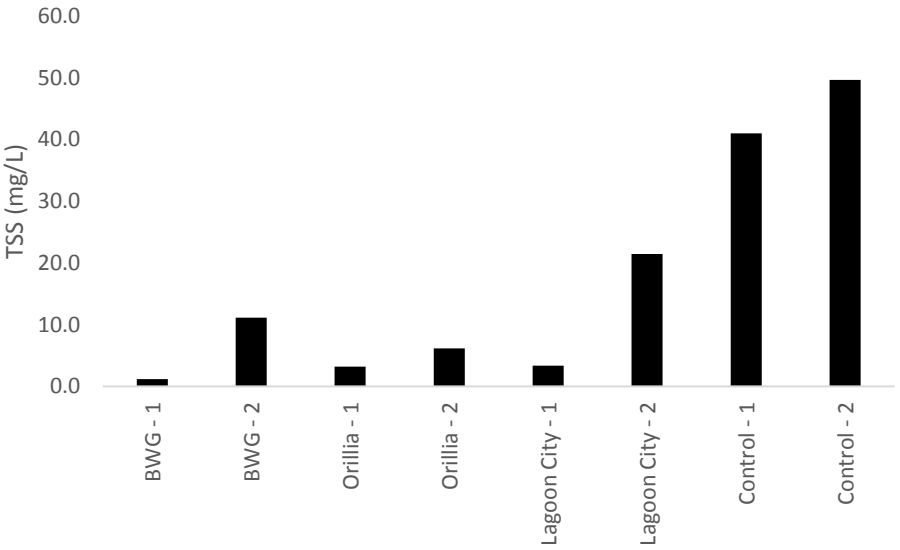

Figure 3.13 - Total suspended solids measurements at each sampling site, where upstream and downstream sites are denoted by ‘1’ and ‘2’.

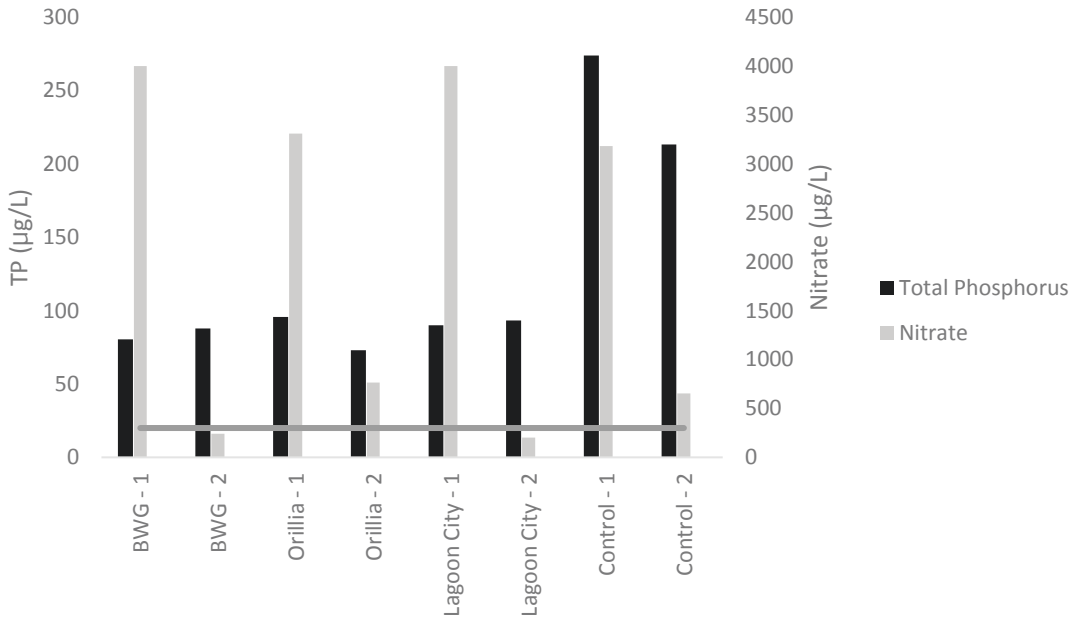

Figure 3.14 - Nutrient concentrations at each sampling site, where upstream and downstream sites are denoted by '1' and '2'. A solid horizontal line denotes total phosphorus guideline (Government of Ontario, 2016).

3.3.2 Presence of PPCPs

PPCP concentrations were not detected at either of the sampling points at the control location. Ibuprofen, and Triclosan were detected at all WWTP locations and Estrone was detected at Orillia and Lagoon City (Figure 3.15). The PPCP concentrations were quite variable amongst WWTP locations, ranging from: Ibuprofen (10.12 to 317.29 ng/L), Estrone (4.20 to 11.90 ng/L), and Triclosan (19.64 to 89.01 ng/L). The highest amounts of Ibuprofen were found at the Orillia sampling points. Estrone was detected in the lowest concentrations compared to the other PPCP in this study. In addition, Estrone was not detected at the BWG sampling points at all. Concentrations of Estrone and Ibuprofen were higher at point one than point two for all WWTP sampling locations. Triclosan followed suit to this trend at the Orillia location, however, these concentrations increased from upstream to downstream at both the BWG and Lagoon City locations.

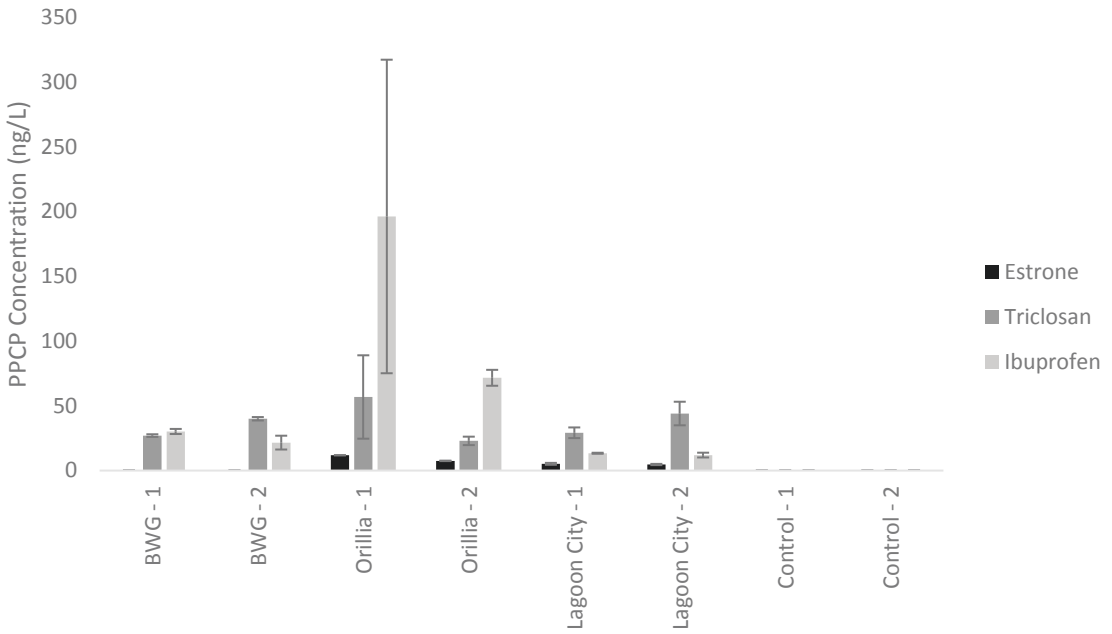

Figure 3.15 - PPCP concentrations (ng/L) at the upstream and downstream sites of each sampling location, where upstream and downstream sites are denoted by '1' and '2'.

3.3.3 Microalgae Parameters

Species richness and diversity were higher downstream than upstream for all WWTP locations, whereas, the opposite occurred at the control location (Figure 3.16). Density was relatively constant between upstream and downstream points for all sampling locations. Percentage of cells belonging to the diatom community was greater downstream than upstream amongst all WWTP locations, whereas, the opposite occurred at the control location (Figure 3.17).

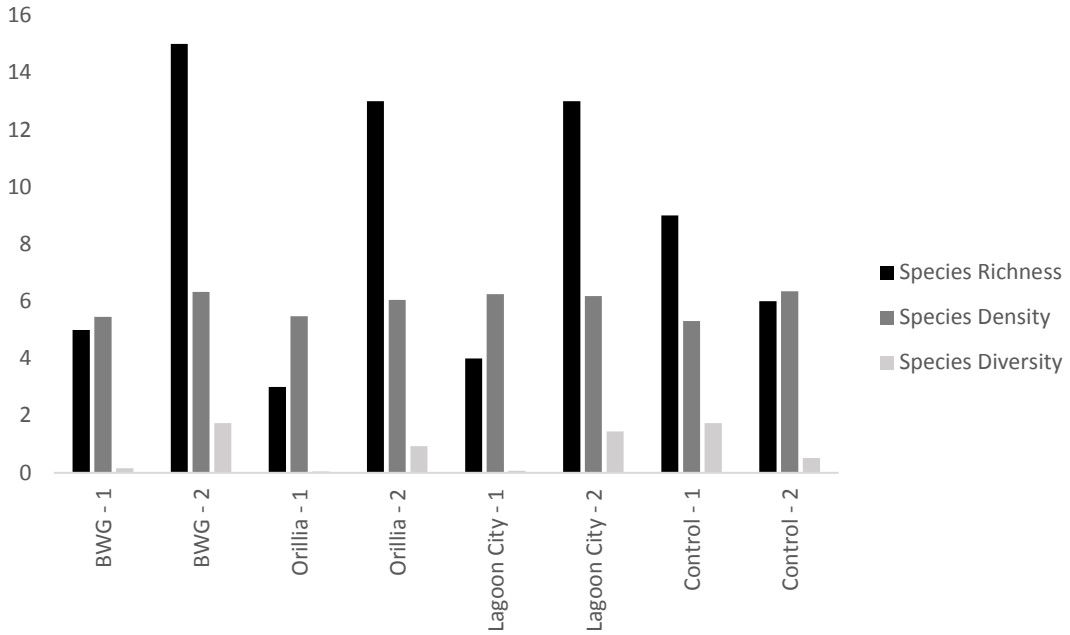

Figure 3.16 - Measurements of algal parameters at both points of each sampling location, where upstream and downstream sites are denoted by '1' and '2'.

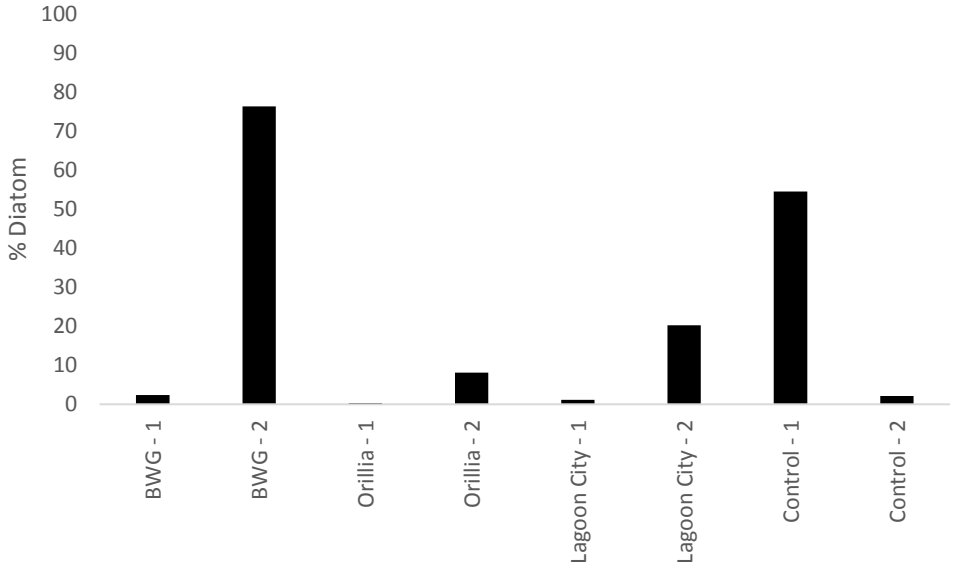

Figure 3.17 - Percentage of cells belonging to the diatom community at all sampling sites, where upstream and downstream sites are denoted by '1' and '2'.

3.3.4 Preliminary Analysis

A correlation matrix (Pearson) was prepared to identify the strength and direction (±) of any relationships between environmental and algal variables (Figure 3.18). The control site was not included in this analysis as most of the microalgae parameters behaved differently at this location from that of the WWTP locations. Strong correlations ($-0.7 \leq r \geq +0.7$) were found between several algal and environmental variables. Species richness positively correlated with site ($r = 0.98$), TSS ($r = 0.71$), and pH ($r = 0.91$), and negatively correlated with nitrate ($r = -0.94$). Species diversity positively correlated with site ($r = 0.94$), TSS ($r = 0.80$), temperature ($r = 0.79$), and pH ($r = 0.97$), and negatively correlated with nitrate ($r = -0.98$). Strong correlations were not found between species density and any of the environmental parameters. Percentage of cells belonging to the diatom community correlated positively with pH ($r = 0.79$) and temperature ($r = 0.83$), and negatively correlated with nitrate ($r = -0.72$). None of the algal parameters showed strong correlations with any of the PPCPs. However, a strong positive relationship was evident between Triclosan and TP ($r = 0.85$).

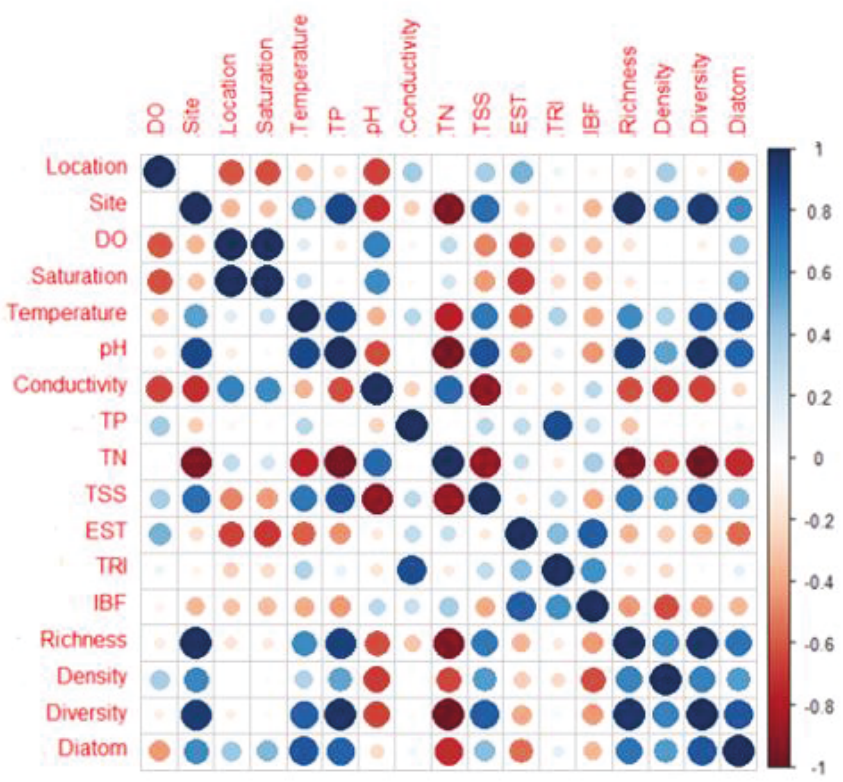

Figure 3.18 - Correlation Matrix plot of environmental and algal variables for the three WWTP sampling locations.

Significance ($\alpha < 0.05$) was found between the following parameters: species richness and site, species richness and pH, species richness and nitrate, species diversity and site, species diversity and pH, species diversity and nitrate, cells belonging to the diatom community and temperature, and TP and Triclosan. The remainder of the relationships exhibited marginal significance ($\alpha < 0.15$).

A Regression Analysis was conducted to explore any correlations between percentage of cells belonging to the diatom community and PPCP concentrations. Significance was not found with Triclosan and Ibuprofen ($p = 0.561$ and $p = 0.379$, respectively). However, Estrone showed a strong negative correlation with marginal significance ($p = 0.071$, $r = -0.846$). Moreover, by designating point one of the Lagoon City sampling location as an outlier, the significance of this correlation became substantially stronger ($p = 0.018$, $r^2 = 0.966$) (Figure 3.19).

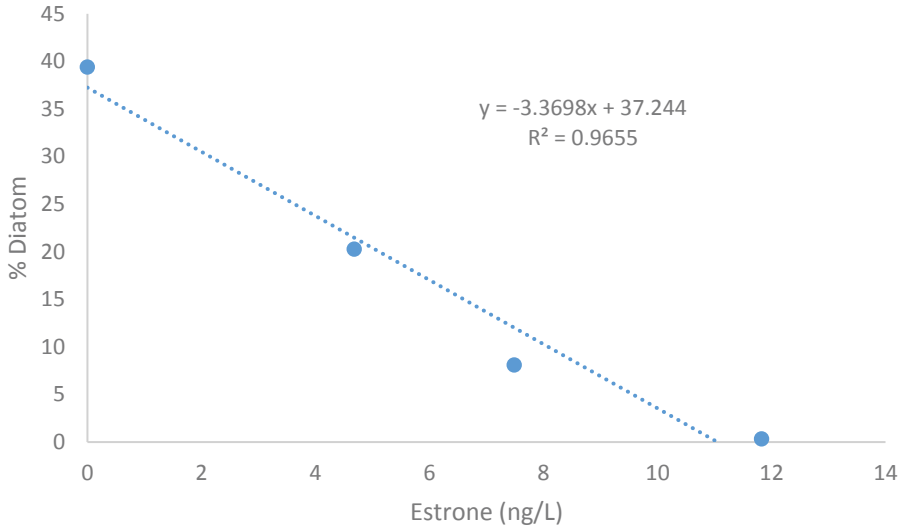

Figure 3.19 - Scatter plot illustrating a linear relationship between Estrone concentrations and percentage of cells belonging to the diatom community.

3.3.5 Ordination Analysis

An ordination analysis was carried out to test the overall relationship between microalgal species and environmental variables including PPCP (Figure 3.20). An overall test of significance showed that the canonical relationship between matrices X (environmental variables) and Y (algal species) is significant ($p < 0.05$ after 999 permutations; permutations of residuals using CANOCO). The first two canonical axes explain 98% and 2% of the response

table's variance, respectively; the first eigenvalue is significant (p = 0.037) and displays strong species-environment correlations (r = 0.973), whereas the second did not exhibit significance (p = 0.212).

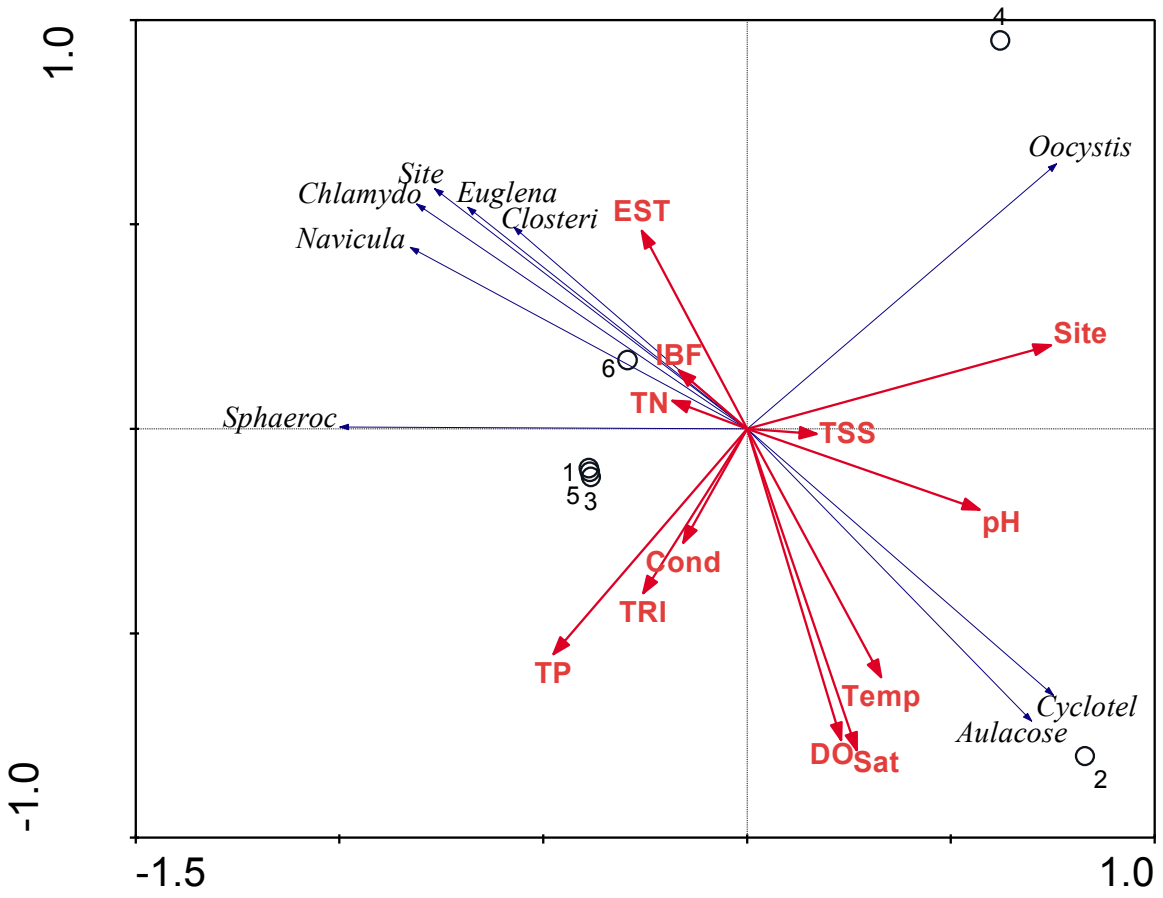

Figure 3.20 - RDA ordination triplot of the field data, inclusive of algal species. Sampling sites are denoted by circles; Environmental variables are denoted by red vectors; Algal species are denoted by blue vectors.

The site locations are labelled one through six (1,2 BWG; 3,4 Orillia; 5,6 Lagoon City) where points 1, 3 and 5 represent site one of each WWTP sampling location and points 2, 4 and 6 represent site two of each WWTP sampling location. Points 1, 3, and 5 are ordinated closer themselves and thus can be expected to have similar values for both environmental and algal parameters. In addition, their relative proximity to TP, Triclosan, and conductivity indicates that these sites would have higher values of these vectors compared to that of the other sites.

While analyzing the relationships between vectors, we look at the angle between the two variables (90º being most unrelated (cos (90) = 0) and 20º being strong and positively correlated (cos (20) = 0.94). An orthogonal relationship exists between site and the following parameters: DO, temperature, and Estrone. This suggests that very little correlation would be expected between site and the aforementioned environmental variables. In contrast, a strongly negative correlation is seen between site and the following variables: Triclosan, conductivity, TP, nitrate, and a species of green algae, *Sphaerocystis*. However, the results also showed a strong positive correlation with the green algae, *Oocystis sp.*. Lastly, it is seen that Estrone and Ibuprofen have strong positive correlations with several algal species: *Navicula sp*., *Chlamydomonas sp.*, *Euglena sp.*, and *Closteriopsis longissima*.

3.4 Discussion

3.4.1 Toxicological Presence of PPCP Concentrations

Several surface water samples were collected in Lake Simcoe outside of three WWTPs and one control location. The samples were analyzed for three PPCPs: Triclosan, Estrone, and Ibuprofen. All three of the compounds were present outside of the WWTPs, however, they were more than a thousand times lower than their respective EC_{50} values, according to the ECOSAR database (US EPA, 2016).

When compared to a previous study in Lake Simcoe, the PPCP values appear to be much higher (Metcalfe, 2014) (Table 3.1). Data on PPCPs collected from the treated effluents discharged into Lake Simcoe in 2014 showed a range between 0.4 to 4.4 ng/L, below detectable limits, and 0.3 to 7.9 ng/L for Ibuprofen, Estrone, and Triclosan, respectively (Metcalfe, 2014). The previous sampling took place in March and December of 2014, whereas, the current sampling was conducted in mid-August of 2016. Previous work examining the seasonal fluctuations of PPCPs suggests that concentrations are highest during the summer months (Kurissery et al., 2012). However, further studies in Lake Simcoe would be required in order to make any conclusive argument of seasonality affecting PPCP concentrations in this region.

Table 3.1 – Concentrations in ng/L of PPCPs outside of three WWTPs on Lake Simcoe from data collected during the winter of 2014 and the summer of 2016.

PPCP	BWG (2014)	BWG (2016)	Orillia (2014)	Orillia (2016)
Ibuprofen	2.8	25.9	0.4	133.9
Estrone	ND	ND	ND	9.7
Triclosan	1.6	33.5	7.3	39.9

The water samples taken from the BWG points did not contain any detectable amounts of Estrone in 2014 or 2016. At a glance, it would be suspected that this could be due to the advanced sand filtration system used in the tertiary treatment. Sand filtration is known to be an excellent process used for the removal of PPCPs. It is reported that up to 99% of many common PPCPs were removed by this process (Soquel Creek Water, 2015). However, this would not explain the amounts of Ibuprofen and Triclosan present in the waters. The major sources of Estrone and other common EDCs found in the wastewater effluents were urine, ingredients of personal care products, detergents, and ubiquitous industrial chemicals (Siegrist et al., 2005). Perhaps the amount of Estrone entering the wastewater treatment facility is in much lower concentrations than that of the other PPCPs. This could only be supported by testing influent samples in addition to effluent samples.

Relatively high concentrations of Ibuprofen were detected outside the Orillia WWTP at both sampling locations. One of the water samples from point one contained Ibuprofen at a concentration of 317.3 ng/L. This WWTP does not contain an extensive tertiary treatment when compared to BWG WWTP, as well as having a much shorter distance between effluent discharge and confluence with the lake. It is suspected that in addition to volume of water, flow rate, etc., dilution is playing a major role in the differences in concentrations of Ibuprofen between points one and two (Table 3.2). As well, the Orillia WWTP services a 230-bed hospital. Ibuprofen is common in the healthcare system, often administered for the relief of pain, fever, and inflammation. As such, it would be appropriate to suggest that a portion of this PPCP present in wastewater effluent would be contributed from this source.

Table 3.2 - Dilution factor of Ibuprofen concentrations at each WWTP location.

Sampling Location	Distance Between Sampling Points (km)	Ibuprofen Concentration Difference Between Points One and Two (ng/L)	Dilution (ng/L per kilometer)
BWG	11	8.59	0.781
Orillia	0.5	124.58	249.16
Lagoon City	2.5	1.40	0.56

The third WWTP for study, located in Lagoon City, showed concentrations of PPCPs at both sampling points similar to that of the other two facilities. The population number being served by the WWTP may play a significant role for the PPCP concentrations. BWG and Orillia have a sizable aeration and settling tanks due to the volume of incoming effluent on a regular basis. To be precise, 72,000 and 17,400 m^3/day of effluent is treated at the Orillia and BWG WWTPs, respectively. Whereas, the Lagoon City WWTP treats a mere 2,000 m^3/day.

Estrone and Ibuprofen were found in higher concentrations closest to the point of discharge than the confluence with the lake outside of all WWTP sampling locations. This suggests that these two PPCPs may degrade or disintegrate into the receiving waters faster, in addition to the dilution effects. Triclosan concentrations behaved in a similar manner at the BWG and Lagoon City locations, unlike the Orillia sampling points. This could potentially be due to the type of substrates at the second points. The substratum at BWG and Lagoon City at the second sampling points was characterized as organic-rich muddy sediment, whereas, the corresponding Orillia point consisted of large sand granules. The sorption of these compounds is thought to be higher for sediment/soil with higher organic content (Yamamoto et al., 2009). In addition, Triclosan exhibits a different sorption capacity from that of Ibuprofen and Estrone, that may be causing this difference in behavior (Hiller et al., 2017; Wu et al., 2009; Yu et al., 2004). Triclosan is reported to have an adsorption-desorption distribution (K_d) ranging from 178 to 264kg/L (Wu et al., 2009). the K_d for Ibuprofen and Estrone is 0.4 to 1.3 kg/L and 3.4 to 3.8, respectively (Hiller et al., 2017; Yu et al., 2004).

The Ordination Analysis also suggested that the first sampling points of each location would be associated with higher values of Triclosan than the second sampling points if sampled repeatedly. Triclosan is found in many of our household products and quite recently, has been banned in the U.S.A. due to its apparent toxicity(Food and Drug Administration, 2016).

However, if Canada were to apply consistent policy, the influx of this substance into our inland freshwaters would be reduced.

3.4.2 Implications on Microalgal Composition

All three PPCPs showed at the very least, marginally negative correlations with the species richness and species density. This indicates that the presence of these compounds is potentially resulting in toxicological impacts on the algal community. This corresponds with several recent reports pertaining to microalgae (Boren, 2015; Morin et al., 2010; Wilson et al., 2003). Due to the significant role that algae play in the freshwater ecosystems, it is possible that these impacts will cascade into the higher trophic levels (Coogan et al., 2007; Parolini, 2010).

A strong negative correlation was found between Estrone and the diatom composition. This relationship is extremely linear when removing only one of the sampling points (Lagoon City, point one). Greater variance amongst the two sampling points for this location was expected to be similar to the other WWTP locations. However, the stable Estrone concentrations between these two points could be due to additional sources of the Estrone compound, via septic systems. The adjoining community to the second sampling point of Lagoon City consisted of older, rural houses and cottages that likely still relied on septic systems for the removal of their grey water.

The analyses revealed strong correlations between diatom composition and sampling points, where diatom composition is greater at the second sampling point than the first sampling point. The green algae*, Sphaerocystis sp.*, was more commonly associated with the first sampling points, whereas, *Oocystis sp.*, was more commonly associated with the second sampling points. Four species of algae were found to be more common with the presence Ibuprofen and Estrone: *Navicula sp.* (Bacillariophyceae), *Chlamydomonas sp.*(Chlorophyceae), *Euglena sp.*(Euglenoidea), and *Closteriopsis longissima* (Chlorophyceae). Other studies in recent literature have begun examining the microalgal community and their sensitivities to PPCP contamination. Antibiotics, antimicrobials, and parabens in particular have been shown to impact diatoms in a manner unique from the rest of the algal community (Morin et al., 2010; Pinckney et al., 2013; Song et al., 2016). Specifically, diatoms showed extreme sensitivity to common bactericides, including Triclosan (Morin et al., 2010). Furthermore, when compared to cyanobacteria, diatoms exhibited heightened sensitivity when exposed to the antibiotic, Tylosin

(Pinckney et al., 2013). Research pertaining to microalgae as indicators of PPCP contamination has only just begun and much work is still required for further understanding of their application in our inland freshwater systems.

3.5 Conclusion

Several PPCPs have been reported entering Lake Simcoe via WWTPs surrounding this water system (Metcalfe, 2014; Metcalfe et al., 2003; Ontario, 2014). This study has confirmed the presence of Ibuprofen, Estrone, and Triclosan in treated effluent as far as the point of confluence with the lake. Seasonality is suspected to play a role in the magnitude of the PPCP concentrations, however, factors effecting these amounts from one point to another appear to be greatly dependent on dilution, substratum composition, and chemical behaviors (i.e. sorption coefficient). Algal parameters varied with the presence of these compounds. Specifically, PPCP concentrations were negatively correlated with species richness and species diversity of the microalgae community. Diatom composition, exhibited a very strong negative correlation with Estrone concentrations. Some species were more sensitive to the presence of PPCPs than others. In this study, many species of green algae were found to be more commonly associated with concentrations of Estrone and Ibuprofen than some of the other diatoms reported. This coincides with previous reports (Morin et al., 2010; Pinckney et al., 2013; Song et al., 2016). As a result, this study indicates that diatoms may have the potential of being used as indicators of pharmaceutical contamination in Lake Simcoe.

4. Conclusions

Diatoms are used as bio-indicators of aquatic health in many freshwater systems (Cattaneo et al., 2004; Dokulil et al., 1997; Lotter et al., 1998). This community of microalgae is sensitive to changes in the aquatic environment, such as acidification, eutrophication, etc. ("European Diatom Database," 2015). The individual and compounding toxicological effects of Ibuprofen, 17-β Estradiol, and Triclosan were explored by examining changes in growth rate of two diatoms, *Asterionella formosa* and *Diatoma tenuis*, by exposing them to varying PPCP concentrations. *A. formosa* and *D. tenuis* were chosen due to their abundance throughout the Lake Simcoe watershed. It was hypothesized that these diatoms would be more sensitive to these PPCPs than what has previously been reported for microalgae belonging to other communities (i.e. Chlorophyceae, Cyanobacteria, etc.). In addition, exposure to a combination of these three compounds would cause compounding toxicological effects which are more severe than the individual compound toxicity. The results of this study support these hypotheses.

A. formosa and *D. tenuis* were more sensitive to the toxicological effects of Ibuprofen and Triclosan than that of previously studied green microalgae (US EPA, 2016). *A. formosa* was more sensitive to the toxicological effects of 17-β Estradiol than the recent reports on green microalgae and other diatoms (Y. Liu et al., 2010; US EPA, 2016). *D. tenuis* was less sensitive than *A. formosa* and the impacts were similar to other reported microalgae when exposed to 17-β Estradiol (Y. Liu et al., 2010; US EPA, 2016). This study suggests that *A. formosa* and *D. tenuis* are more sensitive to the three PPCPs than the other group of microalgae. The range of toxicity was much greater than what is seen in the natural environment, however, this information provides us with an excellent reference for comparison to multiple toxicity exposures. Compounding effects of PPCPs were evident in these two diatoms. Multiple toxicity testing resulted in more severe impacts on the growth of *A. formosa* and *D. tenuis* than individual toxicity testing. These findings are in accordance with previous studies that reported a combined effect of chemical mixture was greater when compared to individual effects (Cleuvers, 2004; Geiger, 2014; Ginebreda et al., 2014; Hunt, 2006). This is particularly important considering current risk assessment protocols are primarily based on the ecotoxicity of single compounds (Ontario, 2014; World Health Organization, 2012), while in the natural system they occur in combination.

Data collected outside of three wastewater treatment plants (WWTPs) that discharge effluent into a creek that feeds into Lake Simcoe, showed that the diatom community varies in response to several water parameters, including PPCPs. Thus, supporting the idea that diatoms are useful bio-indicators of PPCP contamination in a freshwater system, the Lake Simcoe. This was accomplished by assessing the local algal community in relation to several water parameters including temperature, DO_2, pH, conductivity, TP, nitrate, TSS, and three PPCPs (Ibuprofen, Estrone, and Triclosan). Water samples were collected from the point of WWTP effluent discharge and its point of confluence with the lake. It was hypothesized that diatom composition would be reflective of PPCP contamination due to its sensitiveness to these contaminants. The results of this study support this hypothesis.

The occurrence of three PPCPs (Ibuprofen, Estrone, and Triclosan) was confirmed in the nearby areas of the three WWTPs as far as the point of confluence with the Lake Simcoe. The concentrations at the WWTP locations varied significantly from the control location, whereby none of PPCPs in this study were detected. Seasonality is suspected to play a significant role in the amount of PPCPs present outside of these locations, whereby the concentrations are higher in summer than winter (Kurissery et al., 2012; Metcalfe, 2014). PPCP concentrations at the Orillia WWTP varied significantly from the other two WWTP sampling locations, which is largely due to the extremely high concentrations of Ibuprofen at the Orillia sampling location. This compound is present in such high amounts outside of this WWTP is thought to be primarily due to the effluent originating from the hospital. Estrone and Ibuprofen were higher at the sampling points of discharge than the confluence with the lake, likely due to dilution effects. Triclosan did not follow suit to the other two compounds at two of the WWTPs. Triclosan has a very high disassociation constant (K_d) compared to Estrone and Ibuprofen, implying that this compound has a lower affinity to dissociate into the local substratum. This, would allow for the accumulation of Triclosan in these waters, faster than the effects of degradation or dilution to occur.

Diatom composition was negatively affected by the presence of these compounds. Out of the three PPCPs in this study, Estrone showed the strongest impact on the dynamics of the microalgae community. In addition, the algal parameters such as species richness and species diversity were negatively correlated with PPCP concentration in these waters. The ordination

analysis showed some species of microalgae strongly correlated with the presence of these compounds. For example, *Navicula sp.* (Bacillariophyceae), *Chlamydomonas sp.* (Chlorophyceae), *Euglena sp.* (Euglenoidea), and *Closteriopsis longissima* (Chlorophyceae) were found to be more common with the presence of Ibuprofen and Estrone.

The results of this study also showed many other significant relationships. For instance, the sampling points located at the point of discharge outside of each WWTP location were more closely related in regards to water and algal parameters than any of the other sampling points. The green algae, *Oocystis sp.*, was more commonly found at these sites than the points of confluence with the lake. Whereas, the green algae, *Sphaerocystis sp.*, was more commonly found at the points of confluence with the lake, than the points of discharge.

PPCP contamination in Lake Simcoe has only recently been explored (Metcalfe, 2014; Ontario, 2014). As such, a significant gap of knowledge pertaining to the occurrence and consequence of these compounds exist. The results of this study showed that diatoms could function as an indicator of pharmaceutical contamination in Lake Simcoe. The data on the effects of these compounds will help to develop a baseline index of diatoms as bio-indicators of PPCPs in Lake Simcoe. Furthermore, this research will hopefully lead to more studies uncovering the presence of these compounds in our inland waters and the hazards they pose on all aquatic organisms.

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